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Functional Analysis of Two Brassinosteroid Responsive, Putative Calmodulin-Binding Proteins 60 (CBP60S) in Arabidopsis Thaliana

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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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**FUNCTIONAL ANALYSIS OF TWO BRASSINOSTEROID
RESPONSIVE, PUTATIVE CALMODULIN-BINDING PROTEINS 60
(CBP60S) IN *ARABIDOPSIS THALIANA***

(Thesis format: Monograph)

By

Purvikalyan Pallegar

Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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ABSTRACT

Brassinosteroids (BRs) have remarkable ability to increase stress tolerance in plants. Investigations to understand the molecular mechanisms underlying BR-mediated stress tolerance resulted in identification of genes belonging to the family calmodulin binding protein 60 (CBP60). The present study was focused on studying the role of *CBP60f* and *CBP60g* in BR mediated stress tolerance and functional characterization using a reverse genetic approach. The upregulation of *CBP60f* and *CBP60g* by BR and stress noted in publicly available AtGenexpress datasets and by qRT-PCR analysis strongly suggests that these are BR responsive genes and functional analysis of T-DNA insertion mutants showed salt stress related functions in *A. thaliana*. The T-DNA insertion mutants *cbp60f* and *cbp60g* were sensitive to salt stress compared to WT, while *CBP60f* OE lines showed increased salt tolerance. The results of the present study have revealed two new stress related genes, *CBP60f* and *CBP60g* that displayed increased expression in response to BR and salt stress, and also play an important role in conferring salt stress tolerance to plants. *CBP60f* also plays an important role in determining the root length in *A. thaliana*.

KEY WORDS: Brassinosteroids, mutants, reverse genetics, transgenic line, calmodulin, salt stress, root length, *Arabidopsis thaliana*.

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ABBREVIATIONS

μE	microeinstein
ABA	abscisic acid
ANOVA	analysis of variance
ARR	Arabidopsis response regulator
BAK	BRI associated kinase
BES	BRI ems suppressor
BIM	BES1-interacting Myc-like
BIN	Brassinosteroid insensitive
BKI	BRI1 kinase inhibitor
BL	brassinolide
bp	base pair
BR	brassinosteroid
BRF	brassinosteroid response transcription factor
BRI	Brassinosteroid insensitive
BSA	bovine serum albumin
BSU	BRI suppressor
BZR	brassinazole resistant
CaM	calmodulin
CaMBD	calmodulin binding protein
CaMV	cauliflower mosaic virus
CBF	CRT/DRE Binding Factor
cDNA	complementary deoxyribonucleic acid
CML	calmodulin-like calcium-binding protein
Col	Columbia, accession of Arabidopsis
CPD	constitutive photomorphogenesis and dwarfism
DET	deetiolated
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate

DRE	drought-responsive element
DWF	dwarf
EBR	24-epibrassinolide
ET	ethylene
FLS	flavones synthase
GO	gene ontology
GSK	glycogen synthase kinase
GST	glutathione-s-transferase
GUS	β -glucuronidase
h	hour
HS	heat stress
HSF	heat shock factor
Hsps	heat shock protein
JA	jasmonic acid
LEA	late embryogenesis abundant
LRR	leucine-rich repeat
LRR-RK	leucine-rich repeat receptor kinase
mRNA	messenger ribonucleic acid
MS	murashige and skoog medium
NaCl	sodium chloride
PR	pathogenesis-related
RT-QPCR	quantitative real-time reverse transcription polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SA	salicylic acid
SAR	systematic acquired resistance
SDS	sodium dodecyl sulphate
SE	standard error
TAIR	The Arabidopsis Information Resource

TBARS	thiobarbituric acid reactive substances
T-DNA	transfer-deoxyribonucleic acid
UV	ultra violet
VC	vector control
WAK	wall associated kinase
WT	wild type
XTH	xyloglucan endotransglucosyltransferase/hydrolase

CHAPTER 1: INTRODUCTION

1.1 Brassinosteroids

In animals, steroids play an important role acting as hormones. Plants also produce steroidal compounds with growth promoting properties; one class of plant specific steroidal compounds are called brassinosteroids (BRs). In the early 1970s, the word “brassin” was given to an organic extract derived from the pollen of *Brassica napus* that showed growth promoting properties (Mitchell et al., 1970). The, biologically most active BR called ‘brassinolide’ (BL) was isolated from this extract by the USDA (United States Department of Agriculture) scientists (Figure 1.1A) (Grove et al., 1979). In addition to BL, 70 similar compounds were isolated from various plant species, indicating that BRs are ubiquitous in the plant kingdom (Bajguz and Hayat, 2009). BRs occur in both free form, or conjugated to sugars and fatty acids, in almost every part of plants. Pollen and immature seeds contain the highest levels of BR with levels ranging between 1-100 ng g⁻¹ fresh weight, while shoots and leaves have far lower amounts in the range of 0.01-0.1 ng g⁻¹ fresh weight (Bajguz and Tretyn, 2003). Earlier studies showed that exogenous application of BRs to plants increased their growth (Mandava, 1988), but the essential role of BRs in plant growth and development was revealed by studying BR biosynthetic and BR-signaling mutants in *Arabidopsis thaliana* and other plant species (Clouse and Sasse, 1998; Clouse, 2002). These results earned BRs the status of a phytohormone.

1.2 Structure of BRs

BRs belong to the class of polyhydroxysteroids. Structurally BR is derived from the 5 α -cholestane skeleton and is similar to insect and animal steroid hormones. Variations in the BR structure are generated from the type and position of functional groups on the A/B rings and the side chain (Figure 1.1B). These modifications in the A/B rings are produced by oxidation and reduction reactions during biosynthesis. Variations in the A-ring come from α,β -hydroxyl at positions C-2 and C-3, or ketone at position C-3. The most active BRs, such as BL and castasterone (CS), contain C-2 α , C-3 α hydroxyl groups in the A-ring, whereas variations in the B ring, lead to formation of 7-oxalactone, 6-oxo, 6-deoxo, 6-hydroxy and 5-en BRs (Figure 1.1B). The 6-oxo (6-ketone) BRs are most abundant in

Figure 1.1 Structures of brassinosteroids.

A) Structure of brassinolide (BL).

B) Variations in the A and B rings and in the side-chain of naturally occurring plant steroids. The figure has been taken from Bazguz and Tretyn, 2003.



plants, although the biological activity of the 7-oxalactone BRs is stronger than the other forms.

There are eleven different types of BRs according to the structures of the cholestane side-chains with respect to substituents on C-23, C-24 and C-25: 23-oxo, 24*S*-methyl, 24*R*-methyl, 24*R*-methylene, 24*S*-ethyl, 24-ethylidene, 24-methylene-25-methyl, 24-methyl-25-methyl, without substituent at C-23, without substituent at C-24, without substituent at C-23, C-24 (Figure 1.1B). Generally BRs that are not conjugated to a sugar or fatty acid are further classified depending on the alkyl substitutions in side chain as C₂₇, C₂₈ or C₂₉ BRs. The C₂₇ BRs lacking a substituent at C-24 are assumed to be derived from cholesterol. The C₂₈ BRs with either an α -methyl, β -methyl or methylene substituent may be generated from campesterol, 24-epicampesterol or 24-methylenecholesterol, respectively. The C₂₉ BRs with an ethyl group substituent may be derived from sitosterol, and with a methylene at C-24 may come from 24-methylene-25-methyl cholesterol (Fujioka, 1999; Bajguz and Tretyn, 2003). All these variations in BR structure are essential for the proper biological activity of BRs.

1.3 BR Biosynthesis

Research in the last two decades has delineated the pathways involved in BR biosynthesis. Studies on cell suspension cultures of *Catharanthus roseus* fed with isotope labeled putative BR intermediates, and the biochemical analysis of BR deficient mutants have played important roles in determining various steps involved in the core BR-biosynthetic pathways (Sakurai and Fujioka 1997; Fujioka and Yokota, 2003). The sterol-specific (squalene to campesterol), and BR-specific (campesterol to brassinolide), pathways are the two consecutive pathways followed for biosynthesis of BRs. Campesterol, sitosterol and stigmasterol are the three types of sterols generated through the sterol-specific pathway. Condensation and cyclization of mevalonic acid (MVA), the precursor of the terpenoid pathway takes place to initiate sterol biosynthesis by producing squalene. The sterol-specific biosynthetic enzymes generate campesterol along with other

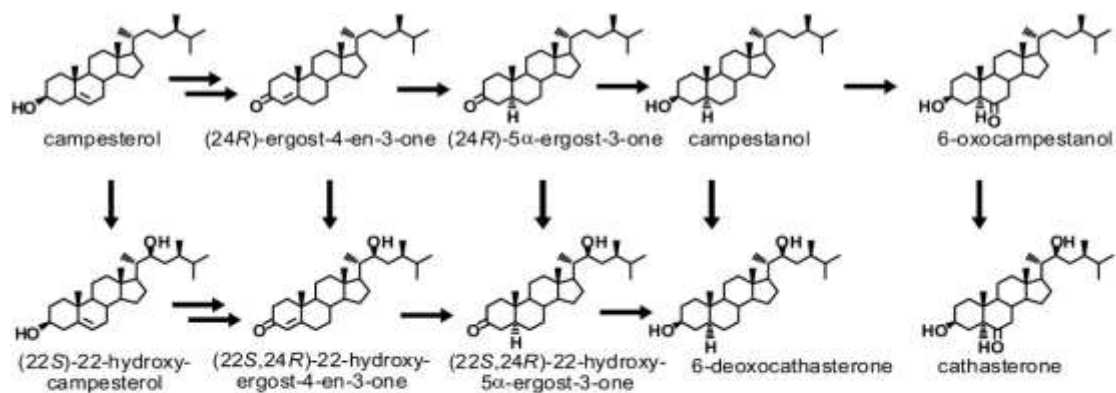
plant sterols by the successive modification of squalene. Campesterol serves as the precursor of all C₂₈ BRs in plants, including BL. The BR-specific pathway in plants is highly networked at multiple steps (Fujika and Yokota, 2003).

Campesterol is converted to campestanol in four conversion steps. Later, BL is generated from campestanol by two parallel biosynthetic routes: the early and late C-6 oxidation pathways (Figure 1.2). Early C-22 oxidation branch that avoids generating campestanol from campesterol acting as a sub-pathway is also linked with the BR-specific pathway (Fujioka et al., 2002). The early C-6 oxidation pathway starts with the conversion of campestanol to 6-oxocampestanol, followed by the enzymatic oxidation of 6-oxocampestanol to cathasterone, teasterone, 3-dehydroteasterone, typhasterol and castasterone. In the late C-6 oxidation pathway campestanol is oxidized at C-22 to generate 6-deoxocathasterone followed by successive oxidation to 6-deoxoteasterone, 3-dehydro-6-deoxoteasterone, 6-deoxotyphasterol, 6-deoxocastasterone and castasterone. The early C-22 oxidation sub-pathway is initiated by generation of 6-deoxocathasterone from campesterol in a four-step process. Later, this sub-pathway follows the late C-6 oxidation pathway to generate castasterone. Another shortcut pathway to generate 6-deoxotyphasterol from campesterol through C-23 oxidation has also been identified (Ohnishi et al., 2006). Finally, the Baeyer-Villiger type of oxidation of C₆-ketone transforms castasterone into BL (Sakurai, 1999).

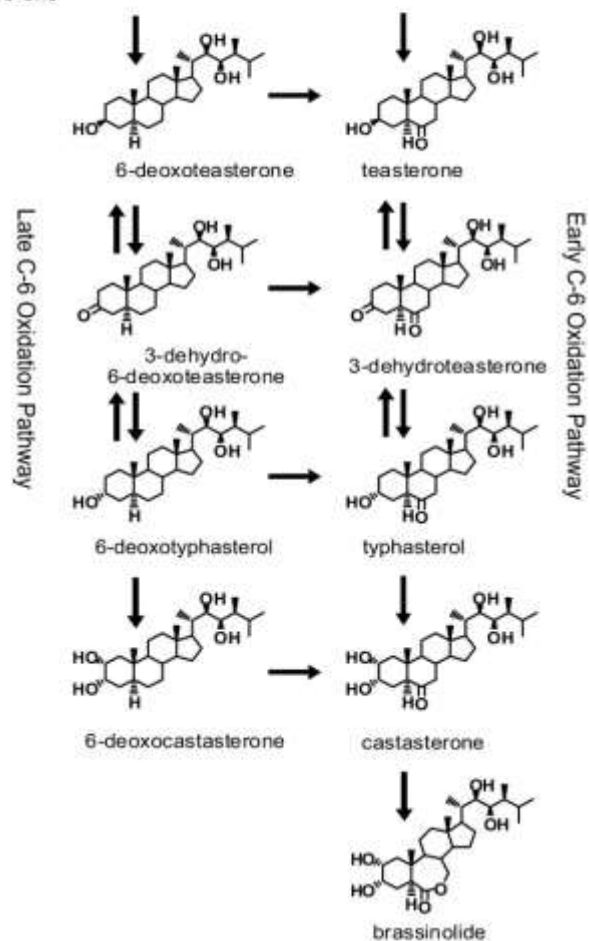
The analysis of BR-deficient mutants primarily in *A. thaliana*, as well as in rice, tomato, and pea helped in the identification of BR-biosynthetic enzymes and their corresponding genes along with their regulatory information (Fujioka and Yokota, 2003, Kwon and Choe, 2005). Phenotypes of BR-deficient mutants include short stature, round curly leaves, reduced fertility, delayed senescence and irregular vascular differentiation (Kwon and Choe, 2005). In many cases the exogenous application of BR reversed the aberrant phenotypes of the BR-deficient mutants to wild type (WT) phenotype (Szekeres et al., 1996; Clouse and Sasse, 1998). A study of light-regulated development in *A. thaliana* resulted in the identification of the dwarf mutant *de-etiolated2* (*det2*) (Chory et al., 1991). The *DET2* gene was found to encode a steroid 5 α -reductase, which is involved in the conversion of campesterol to campestanol or 6-deoxocathasterone (Noguchi et al., 1999;

Figure 1.2 BR biosynthesis pathways.

Enzymes identified in *A. thaliana* are shown. The figure has been adapted from Ohnishi et al. (2006).



Early C-22 Oxidation Branch



Late C-6 Oxidation Pathway

Early C-6 Oxidation Pathway

Fujioka et al., 2002). The defective phenotype in *det2* could be reverted to WT when grown in the presence of exogenous BR, confirming that BR is essential for proper plant development.

Enzymes belonging to the family of cytochrome P450 monooxygenases catalyze the oxidation of BR intermediates; for example, the C-22 and C-23 hydroxylation reactions are mediated by P450 DWF4 (DWARF4) (Choe et al., 1998), and CPD (Constitutive Photomorphogenesis and Dwarfism) (Szekeres et al., 1996), respectively. The C-22 hydroxylation step catalyzed by DWF4 is considered the rate-determining step in the BR biosynthetic pathway. The P450 CPD, belonging to the CYP90 family, catalyzes the C-23 hydroxylation reaction. Both the *A. thaliana dwf4* mutant and the *cpd* mutant are severely dwarfed (Choe et al., 1998; Szekeres et al., 1996). Some BR biosynthetic enzymes like DWF1, DWF4 and CPD contain a calmodulin (CaM)-binding site (Du and Poovaiah, 2005). Removing the CaM-binding site through deletion and site-directed mutagenesis completely inactivated the protein, this suggests, of the likely involvement of Ca²⁺-signaling in the regulation of endogenous BR levels.

1.4 BR signaling

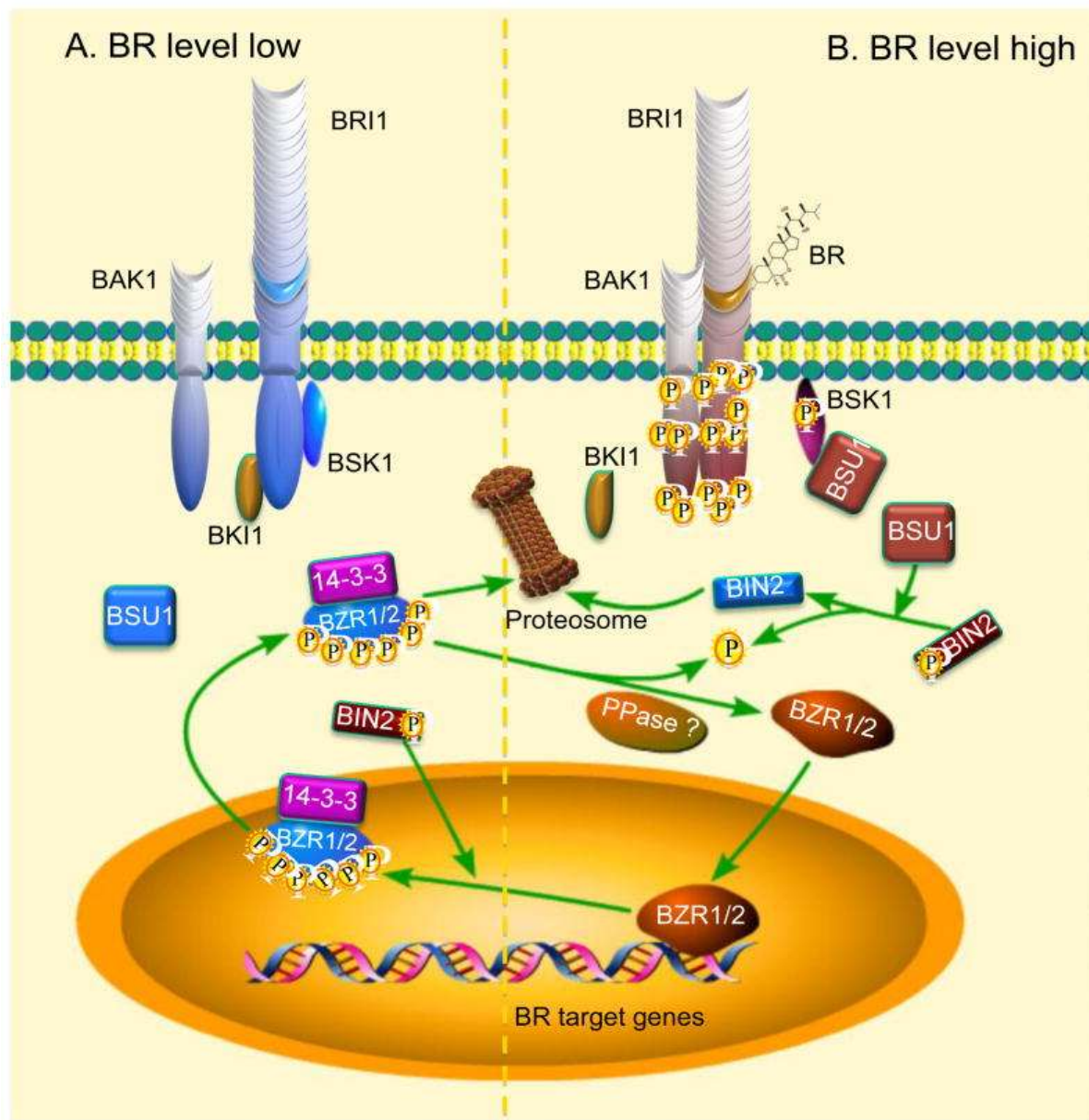
In the last 15 years, BR-signaling has been extensively studied and a number of key signaling components have been identified (Figure 1.3) (Belkhadir and Chory, 2006; Karlova and Vries, 2006; Li and Jin, 2007; Wang et al., 2008; Kim et al., 2009). In animals, steroid hormones are perceived and signal transduction is initiated by direct binding with nuclear receptors. Unlike this mechanism, BRs are perceived by the plasma membrane-localized leucine rich repeat (LRR) receptor like kinase BRI1 (BRASSINOSTEROID INSENSITIVE 1) in plants (Li and Chory, 1997). BR binding to BRI1 causes its dissociation from the BRI1 kinase inhibitor BKI1 and facilitates autophosphorylation as well as association and transphosphorylation between BRI1 and its co-receptor BAK1 (Wang and Chory, 2006). The activated BRI1 phosphorylates BSK1 (BR signaling kinase1), promoting its binding to the phosphatase BSU1 (Kim et al., 2009). Subsequently BSU1 inactivates the negative regulator GSK3-like kinase BIN2 by dephosphorylation, thereby leading to the nuclear accumulation of transcription

factors BZR1 (BRASSINAZOLERESISTANT 1) and BES1 (BRI1-EMS-SUPPRESSOR 1) (also named as BZR2) (Kim et al., 2009). BZR1 and BES1 directly bind to the promoters of BR-regulated genes to affect their expression. BZR1 binds to the CGTG(T/C)G motif found in the promoters of BR biosynthetic genes, *CPD* and *DWF4*, to suppress their expression (He et al., 2005), while BES1 binds to the CANNTG motif (E box) in the *SAUR-AC1* promoter to activate gene expression (Yin et al., 2005). Since numerous physiological processes are regulated by BRs in plants, it is possible that BZR1 and BES1 heterodimerize with other transcriptional factors to regulate transcriptional processes. In the absence of BR, BRI1 is in an inactive form bound to its inhibitor BKI1, and hence BAK1, BSK1 and BSU1 are inactive, while BIN2 is active and phosphorylates BZR1 and BZR2 (BES1), leading to their interaction with 14-3-3 proteins and degradation by proteasome.

1.5 BR functions

Multiple aspects of growth and development in plants are regulated by BR; these include cell division and elongation, seed germination, vascular differentiation, vegetative growth, senescence (Clouse and Sasse, 1998; Sasse, 2003), and flowering-time (Clouse, 2008; Yu et al., 2008). The application of BR in low levels (nM to μ M) showed prominent elongation of hypocotyls, epicotyls, and peduncles of dicots, as well as coleoptiles and mesocotyls of monocots (Clouse and Sasse, 1998). Microscopic examination of BR-deficient and BR insensitive mutants in *A. thaliana* indicated that the dwarf phenotype of these mutants was due to reduced cell size, and not reduced cell number (Sasse, 2003). However, the role of BR in cell division was demonstrated by exogenous application of BR to cell and protoplast cultures. Cell number increase occurred in a culture of synchronously dividing *Chlorella vulgaris* in response to BR treatment (Bajguz and Asami, 2004). In the presence of auxin and cytokinin, application of BR in nM concentrations stimulated cell division by approximately 50% in cultured parenchyma cells of *Helianthus tuberosus* (Clouse and Sasse, 1998). 24-epibrassinolide (EBR), a synthetic BR, when applied with 2,4-D and kinetin to Chinese cabbage protoplasts promoted cell division in a dose-dependent manner, enhanced cluster and colony formation as well as dedifferentiation of protoplasts and the necessary

Figure 1.3 A current model for BR signal transduction pathway. Figure has been taken from Tang et al. (2010).



regeneration of the cell wall before cell division (Clouse and Sasse, 1998). BR also plays an important role in vascular differentiation. Application of nM concentration of BL enhanced tracheary element differentiation in *H. tuberosus* explants (Clouse and Sasse, 1998), whereas an inhibitor of BR biosynthesis, brassinazole inhibited the development of secondary xylem in *Lepidum sativum* (Nagata et al., 2001).

For a long period of time, it is well known that BR has an effect on seed germination and seedling growth. BR promotes the growth of the emerging embryo independent of gibberellin, a phytohormone involved in seed germination and seedling growth (Leubner-Metzger, 2001). Germination inefficiency of gibberellin biosynthetic and signaling mutants was rescued by the application of EBR or BL, suggesting that BR may act in parallel with gibberellin to induce seed germination (Steber and McCourt, 2001). In the presence of abscisic acid (ABA) germination of both BR-biosynthetic (*det2*) and BR-insensitive (*bri1-1*) mutants was inhibited more strongly than WT, indicating that endogenous BR as well as BR-perception is needed to overcome ABA-induced seed dormancy (Steber and McCourt, 2001).

The mechanisms by which BR controls the various physiological processes are not fully known. In one study, the reduced expression of a potent floral repressor *FLC* (*FLOWERING LOCUS C*) in the presence of BRs stimulated flowering (Domagalska et al., 2007). Two Jumonji N/C domain containing transcription factors, ELF6 (EARLY FLOWERING 6) and REF6 (RELATIVE OF EARLY FLOWERING 6), were shown to interact with BES1 (Yu et al., 2008.) ELF6 is a repressor of the photoperiodic flowering pathway; *elf6* mutant displayed an early-flowering phenotype, whereas *ref6* mutant showed higher accumulation of *FLC*, leading to the late-flowering phenotype. Genetic analysis of various mutants (*elf6*, *ref6*, *elf6 ref6* and *bri1 ref6*) also revealed that ELF6 and REF6 function in the BR-signaling pathway (Clouse, 2008).

1.6 The potential of BRs in increasing yield

BRs have remarkable effects on the vegetative growth and seed yield of a number of plant species. Synthetic BRs, such as EBR, 28-homobrassinolide and TS303, can promote growth in a variety of plant species (Khripach et al., 2000), but due to the high

cost of commercial preparations the usage of synthetic BRs in agriculture is limited. Genetic approaches to modulate endogenous BR activity by manipulating genes involved in either BR biosynthesis or signalling have been described (Divi and Krishna, 2009a). Ectopic overexpression of *AtDWF4* under the control of the cauliflower mosaic virus (CAMV) 35S promoter in transgenic *A. thaliana* lead to >2-fold increase in the total number of branches and siliques, 40% increase in inflorescence height and 59% increase in seed yield, as compared to controls (Choe et al., 2001). Similarly, increase in seed yield, branch numbers and height of the inflorescence stem was also seen in maize lines overexpressing a *DWF4* ortholog (Liu et al., 2007). Overexpression of the BR biosynthetic gene *GhDET2* in cotton increased fiber number and fiber length by 22.6% and 10.7%, respectively (Luo et al., 2007). Higher expression of BR-biosynthetic genes during cotton fiber elongation further indicated the role of BR in cotton fiber development (Shi et al., 2006). Other studies also support the notion that genetic modulation of BR biosynthesis or signaling can alter plant architecture. The various effects of BR have potential applications in crop improvement, but the utility of several transgenic plants in the field under different planting conditions remains to be tested (Wang et al., 2007).

1.7 BRs and stress-tolerance

Both biotic and abiotic factors in the environment affect plants; these include drought, high salt, extreme temperatures, heavy metals, wounding, and pathogen and pest attack. In addition to their growth-promoting effects, BRs have also been implicated in plant responses to both abiotic and biotic stresses (Divi and Krishna, 2009a). The stress protective role of BR was demonstrated in plants treated with exogenous BR under standardized stress conditions. In addition, studies with BRsignaling mutants and genome-wide expression data have also provided convincing data for a role of BR in plant stress responses (Divi and Krishna, 2009b).

Response to oxidative stress

Proper growth and development in plants is dependent on cellular homeostasis of reactive oxygen species (ROS). During photosynthesis and respiration ROS are continuously

produced and some integrated and functionally redundant protective mechanisms efficiently control the cellular redox homeostasis. Disruption of these protective mechanisms often generates oxidative stress, leading to the accumulation of ROS that causes damage and death of the cells (Gapper and Dolan, 2006). Cellular homeostasis can be disrupted by almost every kind of environmental stress up to a certain level. Modification of both enzymatic and nonenzymatic antioxidant systems that are involved in maintaining ROS homeostasis and in protecting cells from ROS-induced damage, can be influenced by BRs (Nunez et al., 2003; Ozdemir et al., 2004). Levels of catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) in response to high temperature (40°C) in tomato leaves were increased by EBR treatment (Mazorra et al., 2002). Similarly, treatment with BL increased the activities of SOD, CAT, ascorbate peroxidase (APX) along with levels of ascorbic acid and carotenoids in maize seedlings exposed to water stress (Li et al., 1998). Under osmotic stress conditions, BRs enhanced the activity of CAT and reduced the activities of POD and ascorbic acid oxidase in sorghum (Vardhini and Rao, 2003).

Response to osmotic stress

A number of environmental conditions, e.g., drought, high-salt, freezing, and hypoxia can create osmotic stress to plants. Negative effects of osmotic stress on plant growth can be reduced by BRs. Enhancement of seedling growth in sorghum under osmotic stress conditions (Vardhini and Rao, 2003), and improved nodule development in *Phaseolus vulgaris* (French bean) under water stress condition (Upreti and Murti, 2004) was achieved by the application of BRs. Increase in the survival percentages and growth of seedlings under drought stress conditions was achieved by soaking the roots of *Robinia pseudoacacia* L. in BL prior to planting (Li et al., 2008). These seedlings accumulated higher levels of osmolytes like proline and soluble sugars had higher leaf-water content and greater increases in the activities of antioxidant enzymes like SOD and POD as compared to the untreated seedlings. BR-treated seedlings showed reduced transpiration rate and stomatal conductance under osmotic stress conditions as compared to untreated seedlings (Li et al., 2008).

Response to saline stress

Salt stress has a severe effect on plants by inhibiting seed germination and seedling growth. BR reduced the salinity-induced inhibition of seed germination and seedling growth in rice under salt stress which was concomitant with maintaining of chlorophyll levels and increasing the activity of nitrate reductase (Anuradha and Rao, 2001). EBR application resulted in substantial improvement in seed germination and seedling growth of *Eucalyptus camaldulensis* under salt stress generated by 150 mM NaCl (Bajguz and Hayat, 2009). Nitrogen fixation enhancement and increase in dry matter content, as well as higher seed yield under salt stress was observed in chickpea seeds treated with BL compared to untreated (Ali et al., 2007). Oxidative damage generated by salt stress in salt-sensitive IR-28 rice was also reduced by BR, which was accompanied by increase in the activity of APX (Ozdemir et al., 2004). It was shown that BR can enhance the level of wheat germ agglutinin (WGA), an indicator for environmental stresses, in wheat under salt stress in an ABA independent fashion. A partial growth recovery was observed in wheat treated with BRs and NaCl compared to those of NaCl treatments alone (Shakirova et al., 2002). Exogenous application of BRs conferred tolerance to salinity by altering stress responses in rice variety Pusa Basmati-1 (Sharma et al., 2013).

Response to temperature stress

Under low temperatures stress, BRs has the ability to improve seed germination and seedling growth, this was demonstrated in different plant species, such as rice, maize and cucumber (Bajguz and Hayat, 2009). BR promoted epicotyl elongation and partial recovery of seedling growth in mung bean under chilling stress (Huang et al., 2006). In the same system, it is identified a proteomic analysis that a number of proteins predicted to have roles in cellular growth, ATP synthesis and stress responses to be up-regulated by EBR (Huang et al., 2006). Freezing tolerance of brome grass (*Bromus inermis*) cells was minimally increased by the application of EBR and also shown enhancement in cell viability following exposure to high temperature stress (Wilén et al., 1995). Molecular analysis revealed increased of a subset of ABA-inducible heat-stable proteins in brome grass cells after EBR treatment. EBR increased the levels of *Hsp90* transcripts

during temperature stress but did not induce the expression of dehydrin transcripts unlike ABA. These results indicated that EBR confers stress tolerance to plant cells by mechanisms similar only in part to that of ABA (Wilén et al., 1995). In recent studies by Divi and Krishna (2010), it is shown that *A. thaliana* overexpressing *AtDWF4* under the control of a seed-specific promoter displayed more resistance to low-temperature stress and ABA inhibition of germination.

The basal thermotolerance of *B. napus* and tomato seedlings was increased by the application of EBR (Dhaubhadel et al., 1999, 2002) which correlates with: 1) accumulation of four major classes of heat shock proteins (hsps, hsp100, hsp90, hsp70 and low-molecular-weight hsps in high levels, 2) maintenance of protein synthesis, and 3) increased levels of some translation elongation and initiation factors, as compared to untreated seedlings. The translational machinery appeared to be protected by EBR treatments by limiting the loss of some of the components during heat-stress, and also by promoting the expression of some of the components during post-stress recovery. These lead to a more rapid resumption of cellular protein synthesis following heat stress and a higher survival rate (Dhaubhadel et al., 1999, 2002). The expression levels of genes encoding a mitochondrial transcription termination factor (mTERF)-related protein, *glycine-rich protein 22 (GRP22)*, *myrosinase*, and *3-ketoacyl-CoA thiolase* were substantially changed in EBR-treated *B. napus* seedlings (Dhaubhadel and Krishna, 2008). EBR treatment was also effective in increasing the basic thermotolerance of *A. thaliana* seedlings, however, unlike in *B. napus*, hsp levels were more or less the same in EBR-treated and untreated *A. thaliana* seedlings (Kagale et al., 2007).

Response to pathogen stress

The ability of BR to enhance plant resistance against a broad range of pathogens (viruses, bacteria and fungi) has been suggested in a number of studies. EBR application to barley plants in the field significantly decreased the extent of leaf diseases caused by fungal infection, along with an increase in crop yield (Korableva et al., 2002). A comparison between the fungicide Bayleton and BR treatments indicated that EBR was more effective in promoting resistance against fungal diseases as compared to Bayleton

(Korableva et al., 2002). Similarly, application of EBR to cucumber plants in a field-trial resulted in higher protection against fungal infection, coupled with an increase in the activities of POD and polyphenoloxidase in EBR-treated plants as compared to untreated plants (Korableva et al., 2002). The application of BL increased resistance of tobacco plants against tobacco mosaic virus (TMV), *Pseudomonas syringae* pv. *Tabaci* (Pst) and *Oidium* sp., and of rice against *Magnaporthe grisea* and *Xanthomonas oryzae* pv. *Oryzae* (Nakashita et al., 2003). The authors of this study reported that the level of salicylic acid (SA) and the expression of SA-responsive genes remain unchanged in BL-treated plants, indicating that BR-mediated pathogen stress response is independent of the SA-mediated systemic acquired response (SAR) (Nakashita et al., 2003). In contrast to these results, the SA-responsive pathogenesis-related (PR) genes were expressed at low levels in *cpd*, a mutant impaired in BR biosynthesis in *A. thaliana*, but at significantly higher levels in transgenic plants overexpressing the *CPD* gene (Szekeres et al., 1996), indicating that BR may mediate pathogen resistance, at least in part, through SA-mediated SAR. Limited exposure of *A. thaliana* seedlings to BL also induced the expression *PR1* and *PR2* (Szekeres et al., 1996). While the general conclusion from these observations is that BRs have potential as fungicides, BR concentration, timing and length of BR treatment, and the method of BR application, are important considerations when using BRs as fungicides. For example, a long-term treatment (14 days) of tomato plants with EBR considerably reduced the disease symptoms caused by *Verticillium dahlia* infection, but a short-term EBR treatment (24 h) prior to inoculation with *V. dahliae* had no effect (Krishna, 2003).

Genetic evidence for the role of BR in plant stress responses

Analysis of a rice knock-out (KO) mutant line *Osgsk1* is one of the most convincing evidence for the role of BR in stress tolerance (Koh et al., 2007). *OsGSK1* is the rice ortholog of *BIN2*, a negative regulator of BR-response in *A. thaliana*. *Osgsk1* mutant line was obtained by T-DNA insertion and the seedlings displayed enhanced tolerance to cold, heat, salt, and drought stresses when compared with non-transgenic (NT) segregants. The wilting ratios for KO mutants were about 20, 26 and 36% lower as compared with NT plants after cold, heat, and salt stress, respectively (Koh et al., 2007). In addition, an

elevated expression of abiotic stress-responsive genes was observed in the KO plants under different abiotic stress conditions. A study of ectopic overexpression of *AtHSD1*, a putative steroid regulatory gene from *A. thaliana*, in *B. napus* resulted in transgenic *B. napus* plants that were higher yielding, and more tolerant to salt stress as compared to WT (Li et al., 2007). Transgenic *A. thaliana* overexpressing *AtDWF4* under the control of a seed-specific *oleosin* promoter were more tolerant to cold-stress and ABA-mediated inhibition of germination (Divi and Krishna, 2010). Convincing evidence from these genetic studies along with systematic molecular studies of BR application to *B. napus* and *A. thaliana* seedlings (Dhaubhadel et al., 1999, 2002; Dhaubhadel and Krishna, 2008; Kagale et al., 2007) strongly indicate that BR has an important physiological role in conferring stress tolerance in plants.

Cross-talk of brassinosteroids with other plant hormones

BRs interact with other plant hormones. An additive effect with gibberellin (Mayumi and Shibaoka, 1995) and a synergistic effect with auxin on stem segment elongation (Katsumi, 1991) were exerted by BRs. Synergistic increase in hypocotyl elongation in *A. thaliana* seedlings was shown as a result of co-application of BR and gibberellin, or BR and auxin (Tanaka et al., 2003). Since different plant hormones can regulate similar physiological processes, and cross-talk between different hormones can occur at the level of hormone biosynthesis, signal transduction or gene expression (Nemhauser et al., 2006), it was proposed that BR regulates plant stress responses via cross-talk with other hormones (Krishna, 2003). Indeed, studies of BR effects on the expression of different hormone biosynthetic and/or signalling genes have confirmed this notion (Divi et al., 2010). Increase in the ET levels in *A. thaliana* under normal growth conditions was shown by exogenous application of BL (Arteca and Arteca, 2001). Additive effect of both gibberellins and BRs regulates the endogenous JA levels (Kitanga et al., 2006). JA acts as a signal molecule by integrating the regulation of stress response and development of plants (Schaller et al., 2005). BR induces the expression of *OPR3*, encoding a JA biosynthetic enzyme, which indicates a potential link between BR action and JA biosynthesis (Mussig et al., 2000). SA, a phytohormone involved in biotic stress tolerance, also has important roles in basal and acquired thermotolerance in plants (Clark

et al., 2004; Larkindale et al., 2005). Divi et al. (2010) recently demonstrated that the *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1)*, a protein well recognized for its role in SA-mediated pathogen defense responses, is a critical component of BR-mediated temperature and salt stress tolerance in *A. thaliana*.

It has been assumed for a long period of time that BRs have an antagonistic relationship with ABA in growth and stress responses in *A. thaliana*. The effect of ABA on germination inhibition was more pronounced in BR-deficient mutant as compared to the WT (Steber and McCourt, 2001). Furthermore, transgenic *A. thaliana* plants overexpressing the BR biosynthesis gene *AtDWF4* displayed tolerance to ABA inhibition of germination (Divi and Krishna, 2010). An antagonistic interaction of ABA and BR was observed in the expression of *A. thaliana* BR early response genes *BEE1*, *BEE2* and *BEE3* (*BR Enhanced Expression*) (Friedrichsen et al., 2002). The expression of these genes was repressed by ABA treatment. It is also shown by some biochemical studies that BR signaling outputs as indicated by the phosphorylation status of BES1 and BR-responsive gene expression were inhibited by ABA (Zhang et al., 2009). Although most of the data seems to indicate that ABA and BR mask each other's effects, there exists a report linking BR with increase in ABA levels in the lower plant *Chlorella vulgaris* under stress condition (Bajguz, 2009). Clearly, the interaction between ABA and BR in germination and stress tolerance mechanisms warrants further investigation.

The ability of BRs to protect plants against abiotic stresses has been demonstrated in a number of studies (Dhaubhadel et al., 1999, 2002; Kagale et al., 2007; Koh et al., 2007; Divi and Krishna, 2010), but mechanisms regulating BR-mediated stress tolerance are not well understood. To understand further the molecular mechanisms of BR-mediated stress tolerance, BR-responsive transcriptome under no stress and stress conditions (1 and 3 h exposure at 43°C) was determined in Arabidopsis using microarray analysis by former lab members (Divi, 2008; Rahman, 2010). Within this dataset, subsets of putative calcium-signaling genes were identified, including two genes encoding calmodulin-binding proteins 60 (CBP60). The two proteins, CBP60h and CBP60e belong to an eight-member protein family in Arabidopsis, consisting of CBP60a-h. *CBP60h* and *CBP60e* were analyzed for their functions using an array of genetic and genomic approaches

(Rahman, 2010; Krishna and coworkers, unpublished data). Both genes were found to be responsive to BR and to have a role in salt tolerance. In addition both genes also have a role in plant defense responses (Wang et al., 2011; Krishna and coworkers, unpublished data).

1.8 Calcium, Calmodulin (CAM) & CBP60 family

Calcium is important in the activation of biotic and abiotic stress-related signalling cascades (Kiegle et al., 2000). Ca^{2+} is a ubiquitous secondary messenger, which mediates stimulus-response coupling in the regulation of diverse cellular functions. In plants, cytosolic Ca^{2+} concentration can be rapidly elevated in response to a variety of stimuli such as light, plant hormones, touch, abiotic stresses and pathogen elicitors, which then quickly returns to basal level causing Ca^{2+} spike. The transient Ca^{2+} elevations are sensed by proteins such as calmodulins (CaMs), CaM-like proteins (CMLs), calcium dependent protein kinases (CDPKs), and calcineurin B-like proteins (CBLs) (Figure 1.4A) (Day et al., 2002; Yang and Poovaiah, 2003).

CaM is a multifunctional protein in all eukaryotes, consisting of two globular domains, each with two Ca^{2+} -binding EF-hand motifs. Upon binding to Ca^{2+} , the hydrophobic surfaces in each globular domain are exposed which then interact with the characteristic amphiphilic structure called CaM-binding domain (CBD) present in CaM-binding proteins (CBPs or CaMBP) (Snedden and Fromm, 2001; Reddy et al., 2002). This interaction leads to conformational changes in CBPs and modulation of their activity. (Figure 1.4B) Thus, CaM couples Ca^{2+} signals to changes in the activities of downstream proteins via direct interaction. The repertoire of CBPs in plants includes many structurally and functionally unrelated proteins that are involved in various biological processes such as regulation of metabolism, morphogenesis, cell division, cell elongation, ion transport, gene regulation, cytoskeletal organization and stress tolerance (Yang and Poovaiah, 2003). Examples of CBPs involved in stress tolerance in Arabidopsis include ACA4, a vacuolar Ca^{2+} -ATPase (Geisler et al., 2000), some members of the CaM-binding transcription activator (CAMTA) family (Reddy et al., 2002), CaMBP25, a

nuclear localized protein, (Perruc et al., 2004), and CBP60g and CBP60h (*SARD1*) (Wang et al., 2011).

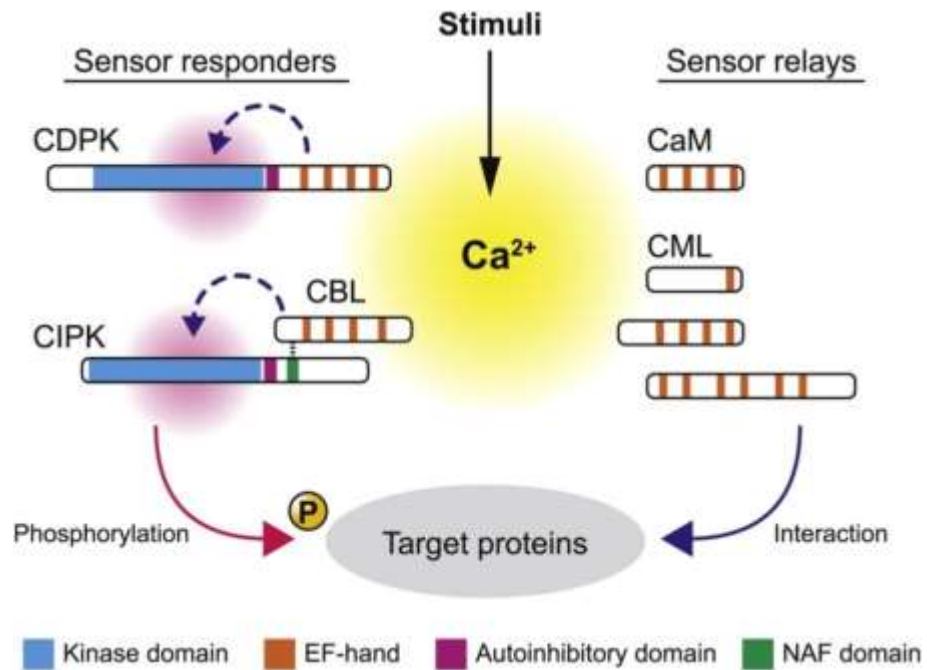
CBP60 refers to a protein family members of which are putative or actual calmodulin-binding proteins expressed by various plant species. In *Arabidopsis* there are eight different genes (*CBP60* 'a' to 'h'), among them *CBP60g* and *CBP60h*, also referred as *SARD1* (*systemic acquired resistance deficient 1*), have been implicated in plant defense responses and accumulation of salicylic acid (SA) (Wang et al., 2011). Both these proteins were shown to bind DNA through their highly conserved central region, which exhibits no sequence similarity to other known DNA-binding proteins, and regulate expression of specific genes. Thus, the CBP60 family likely represents a plant-specific family of transcription factors, with CaM-binding ability (Wang et al., 2011). Recently, CBP60g was also shown as a positive regulator for drought tolerance (Dongli et al., 2012).

Figure 1.4: Calcium sensing and Calmodulin

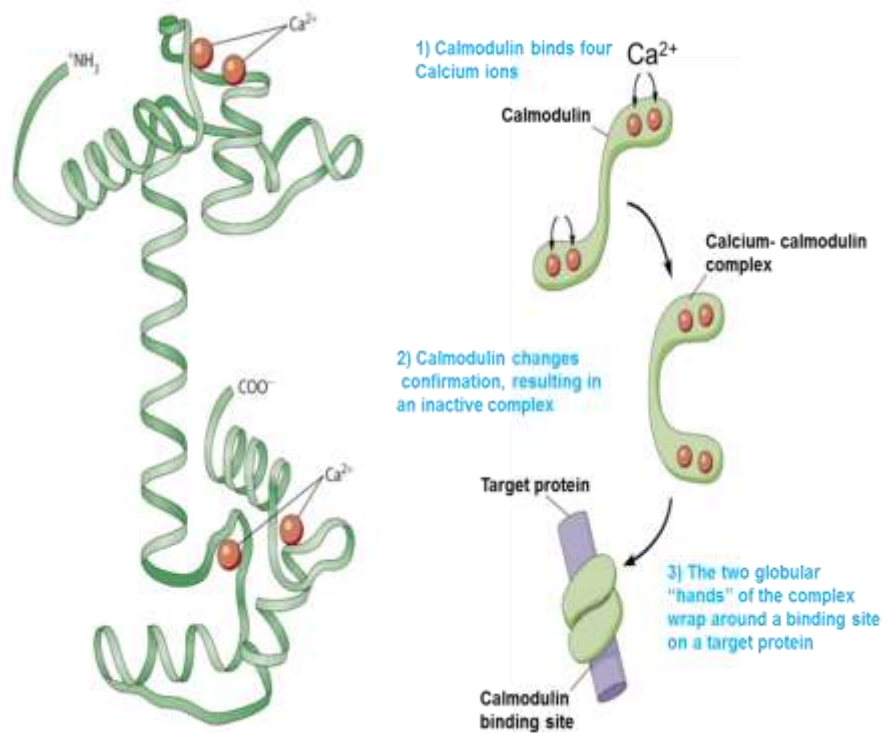
A) Calcium sensing: Ca^{2+} elevations are sensed by proteins such as calmodulins (CaMs), CaM-like proteins (CMLs), and calcium dependent protein kinases (CDPKs), calcineurin B-like proteins (CBLs).

B) Structure and function of Ca^{2+} -CaM complex.

Figures are taken from Day et al. (2002); Yang and Poovaiah 2003.



A



B

1.9 Rationale of the study

The role of BRs as a phytohormone is unique, they work as growth promoting agents, as well as protect plants from both abiotic and biotic environmental stresses. But our knowledge of the molecular mechanisms underlying BR-mediated stress tolerance in plants was not well understood. In this aspect of investigation, a novel family named CBP60 with putative or actual calmodulin binding activity was found and two out of eight members from that family were already shown to be BR responsive and having some role in salt tolerance. To determine if other members of the *CBP60* family are BR- and stress- responsive, I searched the public database dubbed AtGenExpress (datasets from a collaborative microarray project based on Affymetrix ATH1 arrays) and found *CBP60g* and *CBP60f* to be responsive to BR and salt stress. Additionally, *CBP60g* is also responsive to cold and osmotic stresses. These genes are maximally expressed in leaf tissue. **Considering that *CBP60f* and *CBP60g* appear to be BR-responsive and maximally upregulated by salt stress according to the datasets in AtGenExpress, I hypothesize that these two genes play an important role in BR-mediated salt stress tolerance.** The objectives of the present study are focused on the role of these two genes in salt stress which includes:

- To validate the responsiveness of *CBP60f* and *CBP60g* to BR and salt stress by qRT-PCR analysis.
- To confirm homozygous T-DNA insertion mutants for gene knockout (KO) using RT-PCR.
- Generate overexpressing (OE) transgenic lines.
- Carry out detailed phenotypic analysis of KO mutants and OE lines, relative to WT, under no-stress and salt stress conditions.

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant materials and growth conditions

A. thaliana ecotype Columbia (Col-0) was used in all experiments. One confirmed homozygous T-DNA insertion line (*CS860840*) for *CBP60f* (AGI code: AT4G31000) and two confirmed homozygous T-DNA insertion lines (*SALK_023199c* and *CS860821*) for *CBP60g* (AGI code: AT5G26920) were obtained from the Arabidopsis Biological Resource Center (ABRC; Ohio State University, Columbus, OH). According to the sequence-viewer in TAIR (<http://www.arabidopsis.org/>), the T-DNA in *CS860840* is inserted in the sixth exon of *CBP60f*. *SALK_023199c* has the T-DNA inserted in the third exon of *CBP60g*, while *CS860821* has the insertion in the fifth exon. All studies involving *cbp60f* mutant were carried out using the homozygous line *CS860840*, while two mutant lines *SALK_023199c* and *CS860821*, termed as *cbp60g-1* and *cbp60g-2* respectively, were used for *cbp60g*.

Plants were grown either on soil or on 0.5X Murashige and Skoog (MS) (Sigma, St. Louis) medium supplemented with 1% phytablend (Caisson labs, USA) and 0.5% sucrose. Seeds were surface sterilized by sequentially soaking in 75% ethanol, rinsing 4-5 times in sterile water, soaking in 20% (v/v) commercially available bleach for 12 minutes with stirring, and then rinsing 4-5 times with sterile water. To encourage synchronized germination, seeds were placed in sterile 1.5 mL eppendorf tubes with 250 μ L of H₂O and kept for 3 days in the dark at 4°C prior to planting in soil or MS plates. Seeds were germinated and grown inside growth chambers set at 22°C under a 16/8 h light-dark cycle at 80 μ Em^{-2 s⁻¹}.

2.2 RT-PCR analysis

Leaf tissue of T-DNA insertion lines *CS860840*, *SALK_023199c* and *CS860821* was checked for *CBP60f* and *CBP60g* transcript expression by RT-PCR. RNA was extracted from frozen leaf tissue collected from 21-day-old WT and T-DNA insertion lines using SV total RNA Isolation System (Promega, Madison, WI). cDNA was prepared from 1 μ g of total RNA using QuantiTect Rev. Transcription Kit (QIAGEN). *CBP60f*-specific primers (F5'-ATGGAAAATTCTATGAACAACAGAGG -3' and R5'-

TTAGTCGATCTCCACAATTTGAGG-3') and *CBP60g*-specific primers (F5'-ATGAAGATTCGGAACAGCCCTAGTT-3' and R5'-TTACAAGCCTTCCTCGGATTTCT-3') were used to amplify fragments from the cDNA preparations. The amplification of *ACTIN* (F5'TGCTCTTCCTCATGCTATCC3' and R5'ATCCTCCGATCCAGACACTG3') was used as loading control. The amplification conditions were: initial denaturation step at 94°C for 5 min followed by 25 cycles for *ACTIN*, and 30 cycles for both *CBP60f* and *CBP60g* of denaturation at 94°C for 30 s, annealing at 58°C for 45 s and extension at 72°C for 1 min, and final extension at 72°C for 7 min.

To analyze organ-specific *CBP60f* and *CBP60g* expression, total RNA isolated from leaves, stem, roots of 17-day-old WT plants, flower tissues of 21-day-old plants, and seeds of 8 weeks old plants were subjected to RT-PCR analysis. RNA isolation and cDNA preparation was carried out as described above. PCR was carried out with gene specific primers (*CBP60f*- F5'- CTCCATGGAAAATTCTATGAACAACAGAGG -3' and *CBP60f*- R5'-GGTCTTAGTCGATCTCCACAATTTGAGG-3', and *CBP60g*- F5'-CTCCATGAAGATTCGGAACAGCCCTAGTT-3' and *CBP60g*- R5'-GGTCTTACAAGCCTTC CCTCGGATTTCT-3'). *ACTIN* was used as a loading control and the amplification conditions were same as above.

2.3 Salt stress treatment

Seeds of WT and knockout (KO) mutants (*cbp60f* and *cbp60g*) were allowed to germinate on MS medium (control) and MS medium containing 100 mM, 150 mM and 200 mM NaCl concentrations. Radicle emergence was observed and scored daily for up to 6 days, and percent germination was determined for each genotype.

Salt tolerance assay to test the effect of long-term salt stress was done on 3 weeks old plants according to Magnan et al. (2008). WT, KO mutants and overexpression plants were grown in square pots (8.48 cm x 8.48 cm x 8.26 cm; 03.50 STD TL TW from Dillen Products) filled with an equal amount of soil in a growth chamber (22°C, 16/8 h, 80 $\mu\text{E m}^{-2} \text{ s}^{-1}$ of light). Five plants were grown in each pot for 21 days and then irrigated with equal volume of either water (control) or 150 mM NaCl solution every 3 days. For maintaining a homogeneous experimental condition, approximately 1.5L of water or 150

mM NaCl solution was directly put on trays containing the soil-pots. Salt stress was given for 4 weeks, and the resulting symptoms were monitored on alternative days. Plants with intact green leaves in the middle of the rosette after the treatment were considered as survivors, and plants exhibiting complete senescence of the entire body were considered as dead. The survival percentages were determined after 4 weeks. Experiments were repeated three times. The number of plants tested for each genotype varied from 15 to 25 in each experiment.

2.4 Brassinosteroid treatment of plants

Plants were exposed to BR for either 3 weeks (long term treatment) or for up to 24 hrs (short term treatment). For the long term treatment, WT seeds were surface sterilized and stratified for 3 days in the dark at 4⁰C and allowed to germinate on MS medium (control) or MS medium containing 0.1 μ M brassinolide (BL). Aerial tissues were collected from 3 weeks old plants for transcript analysis. In the short term treatment, leaves from 3 weeks old WT plants grown on MS media were allowed to float on liquid MS medium containing 0.1 μ M BL, and tissue samples for RNA extraction were collected at 0, 1, 3, 6, 12 and 24 hr time intervals of the treatment.

2.5 RNA isolation and real-time quantitative RT-PCR (RT-qPCR)

To analyze the expression of *CBP60f* and *CBP60g* transcripts in response to salt and BR treatments, leaf tissues were collected from WT plants treated with 150 mM NaCl and 0.1 μ M BL as described in section 2.3 & 2.4, and quickly frozen in liquid N₂ and stored at -80⁰C. RNA extraction was done using SV total RNA Isolation System (Promega, Madison, WI) and QuantiTect Rev. Transcription Kit (QIAGEN) was used for cDNA synthesis. PCR reaction was carried out in a Rotor Gene-3000 thermocycler from Corbett Research (Sydney, Australia) using 0.1X SYBR-Green I (Invitrogen, Carlsbad, CA). PCR conditions for *CBP60f* analysis included an initial denaturation step at 94⁰C for 4 min followed by 35 cycles of denaturation (15 s at 94⁰C), annealing (30 s at 58⁰C) and extension (35 s at 72⁰C and 15 s at 83⁰C). With the exception of annealing, which was for 30 s at 60⁰C, the same conditions were maintained for analysis of *CBP60g* expression.

CBP60f transcript expression in leaf tissues of 14-day-old WT and OE transgenic plants was examined by RT-qPCR using the same conditions as noted above. *CBP60f*-specific primers (F5'-TCACGGAGGAAATTCGAGCG-3' and R5'-CTGTTACCAAACCCGCCTGA-3') and *CBP60g*-specific primers (F5'-TCGCTCACGATTGAAGCTGT-3' and R5'-CACAAGCGGAGAACCATCCT-3') were used in this analysis. All reactions were performed in triplicate for each biological replicate. Values were normalized using *Ubiquitin* as the internal control, and fold change in the expression level was determined according to $2^{-\Delta\Delta CT}$ described by Livak and Schmittgen (2001). Three biological replicates were used in the analysis to determine relative expression of *CBP60f* and *CBP60g*.

2.6 Protein sequence alignments and promoter analysis

Full length amino acid sequences of the CBP60 protein family members were obtained from the National Center for Biotechnology Information (NCBI) and the *A. thaliana* Information Resource (TAIR) and multiple sequence alignment was done using Multalin at default settings (<http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html>). *CBP60f* and *CBP60g* promoter sequence analysis was done using PLACE (a database of plant cis-acting regulatory DNA elements, <http://www.dna.affrc.go.jp/PLACE/>). The promoter sequence was searched for the presence of motifs identical with or similar to the previously reported motifs in the PLACE database by Signal Scan Search.

2.7 In silico expression analysis using AtGenExpress Visualization Tool (AVT)

The developmental, and hormone and stress-induced expression patterns of *CBP60f* and *CBP60g* were derived from the publicly available AtGenExpress datasets, which were developed through a collaborative microarray project using 79 different *Arabidopsis* samples in triplicate to generate expression data. These data were accessed using AtGenExpress Visualization Tool (AVT) (<http://www.weigelworld.org/resources/microarray/AtGenExpress/>) and absolute expression values retrieved by AVT were used to develop bar diagrams.

2.8 Expression constructs and generation of transgenic plants

Gateway cloning technology was used in the preparation of overexpression constructs for *CBP60f*. Primers (F 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTC CATGGAAAATTCTATGAACAACAGAGG-3' and R 5'GGGGACCACTTTGTACAA GAAAGCTGGGTCTTAGTCGATCTCCACAATTTGAGG-3') were designed according to the gateway primer design protocol to include the full-length coding sequence (CDS) of *CBP60f* available through GenBank (Accession number: NC_003075). *CBP60f* cDNA was amplified using these primers and Phusion™ High-Fidelity DNA Polymerase (New England Biolabs), and the resulting 1.68 kb fragment was introduced into the entry vector pDONR™221 (Invitrogen) using BP clonase II (Invitrogen) according to the manufacturer's instructions (Figure 2.1). The target fragment in the entry clone was sequenced and then transferred by recombination to pEarlyGate100 and pEarlyGate104 (Earley et al., 2006). The resulting overexpression constructs were used in generating transgenic *A. thaliana* plants, and in examining the subcellular localization of the protein in leaves of *Nicotiana benthamiana*, respectively. Following another round of sequence verification, the overexpression constructs were introduced into *Agrobacterium tumefaciens* GV3101 by electroporation. *A. thaliana* was transformed using the floral dip method (Zhang et al., 2006). Seeds from T₀ transformants were allowed to germinate on soil and the primary transformants (T₁) were selected by spraying the herbicide Basta (glufosinate) on 3-day-old seedlings. Basta resistant transgenic T₁ plants were self-fertilized to yield T₂ seeds. Homozygous lines were isolated by screening T₂ seeds for 100% survival against Basta.

2.9 Subcellular localization analysis of *CBP60f*

The *Agrobacterium* culture harboring either the *CBP60f* overexpression construct in pEarlyGate104 or the empty pEarlyGate104 vector (control) was infiltrated into leaves of 21-day-old *N. benthamiana* plants according to the protocol described by Sparkes et al. (2006). After 72 hours of inoculation the infiltrated area of leaves were taken and slides were made for microscopic examination. Slide preparation was done by placing the sliced leaf on a single microscope slide with a drop of water between the slide and leaf and another drop of water on the top surface of the leaf for adhesion of the cover slide. Leica

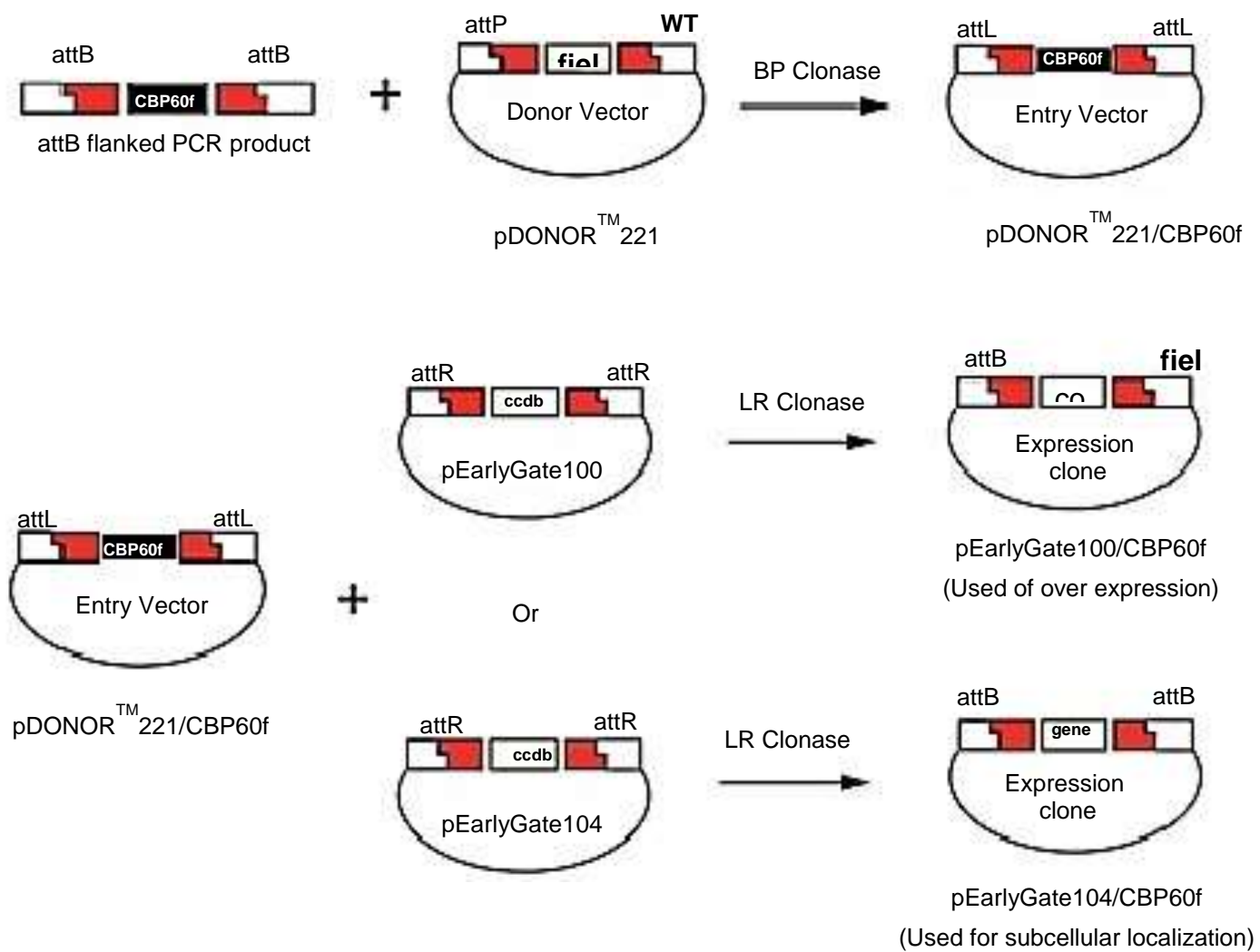
DMIRE2 confocal microscope with either a 10X Dry or 63X W Corr objective was used to observe the individual cells. Fluorescent proteins were excited with an Argon-Krypton laser. Simultaneously, data for different color channels were collected. A charge-coupled device camera was used to collect the images and that were edited using the Microsoft Photo Editor Software package.

2.10 Root length assay

Seeds of WT, KO and OE lines were surface sterilized and grown in soil-pots in a growth chamber maintained at 22°C under a 16/8 h light-dark cycle at 80 $\mu\text{Em}^{-2} \text{ s}^{-1}$. The root system of 11 day old seedlings was gently pulled out of soil and the root length was measured using a standard ruler.

Seeds of WT, mutant *cbp60f* and *CBP60f* OE lines (*PKf2*, *PKf3*, *PKf4*, and *PKf6*) were allowed to germinate on vertically placed MS medium plates containing different salt concentrations (0 mM, 100 mM, 150 mM, and 200 mM NaCl) and root length was measured in 7 -day-old plants. All the experiments for root length assay were repeated three times. For statistical analysis, student's t-test and one-way annova were used to determine the significance difference.

Figure 2.1 Gateway cloning strategy for generating *CBP60f* overexpression constructs.



CHAPTER 3: RESULTS

3.1 *CBP60f* and *CBP60g* genes are upregulated by BR and salt stress

CBP60 gene family members were identified in a microarray study of BR mediated gene expression under nonstress and stress condition (Divi 2009). Previously, salt stress-induced transcript expression was noted for *CBP60e* (Prasad, Pallegar and Krishna, unpublished) and *CBP60h* (Rahman 2011). To see if the expression of *CBP60g* and *CBP60f* was affected by environmental stresses, the AtGenExpress dataset was availed using AVT. Both genes were found to be expressed predominantly in leaves and *CBP60f* was also expressed at high levels in roots. These genes were responsive to osmotic, cold and drought stress in shoots (Figure.3.1A and 3.2A), and maximally responsive to salt stress in roots (Figure.3.1B and 3.2B) Salt responsiveness of the two genes was confirmed by qRT-PCR in shoot tissue of 21-day-old WT seedlings treated with 150 mM NaCl for 2 weeks. An approximate of 2.9 and 4.8 -fold increase in *CBP60g* and *CBP60f* transcript expression respectively, was noted relative to the unstressed control (Figure.3.3A).

Since microarray analysis and subsequent studies showed that *CBP60* gene members are responsive to BR, the same responsiveness was analyzed for *CBP60g* and *CBP60f* both through *in silico* studies (AtGenExpress dataset) and qRT-PCR was availed using AVT. Of the various hormones tested in studies leading to the AtGenExpress datasets, BR (10 nM BL) maximally increased the expression of *CBP60f* at 3 h of treatment, and of *CBP60g* at 1 and 3 h of treatment. The other hormones also showed some effects, but BR impacted the maximum increase in expression (Figure 3.1C and 3.2C). BR-responsive expression of the two genes was further confirmed by qRT-PCR in 21-day-old WT seedlings grown on MS plates containing 0.1 μ M BL (long-term treatment). BR increased *CBP60g* and *CBP60f* expression by approximately 2.9 and 1.4 -fold, respectively, relative to control (Figure 3.3B) To check the effect of BR in a short term treatment, leaves were taken from 21- day-old WT seedling grown on MS medium alone and floated in liquid MS medium containing 0.1 μ M BL for varying times. The expression of *CBP60f*

Figure 3.1: *In silico* analysis of *CBP60f* expression using AVT.

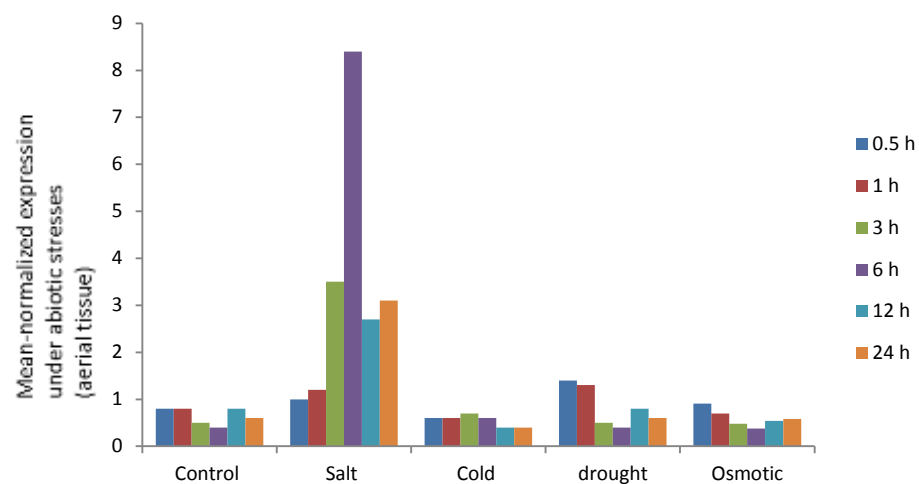
The mean-normalised and absolute expression values for *CBP60f* were retrieved by AVT and plotted.

A) *CBP60f* expression in response to time course exposure to different abiotic stresses in aerial tissues.

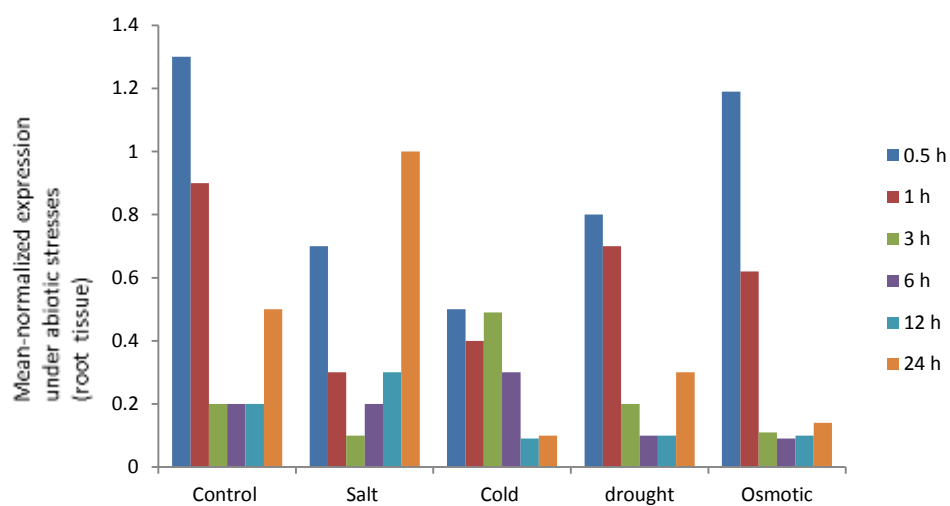
B) *CBP60f* expression in response to time course exposure to different abiotic stresses in root tissues.

C) *CBP60f* expression in response to time course to different hormones.

A.



B.



C.

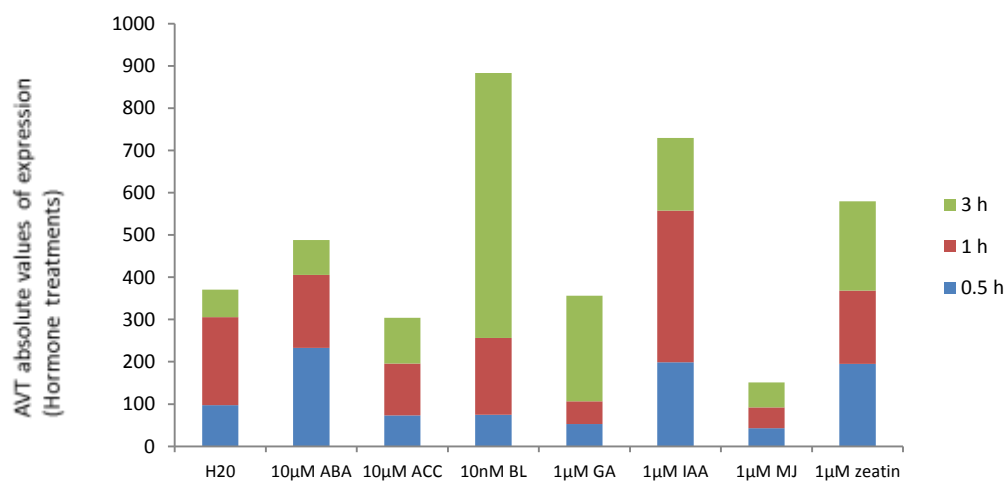


Figure 3.2: *In silico* analysis of *CBP60g* expression using AVT.

The mean-normalised and absolute expression values for *CBP60g* were retrieved by AVT and plotted.

A) *CBP60g* expression in response to time course exposure to different abiotic stresses in aerial tissues.

B) *CBP60g* expression in response to time course exposure to different abiotic stresses in root tissues.

C) *CBP60g* expression in response to time course to different hormones.

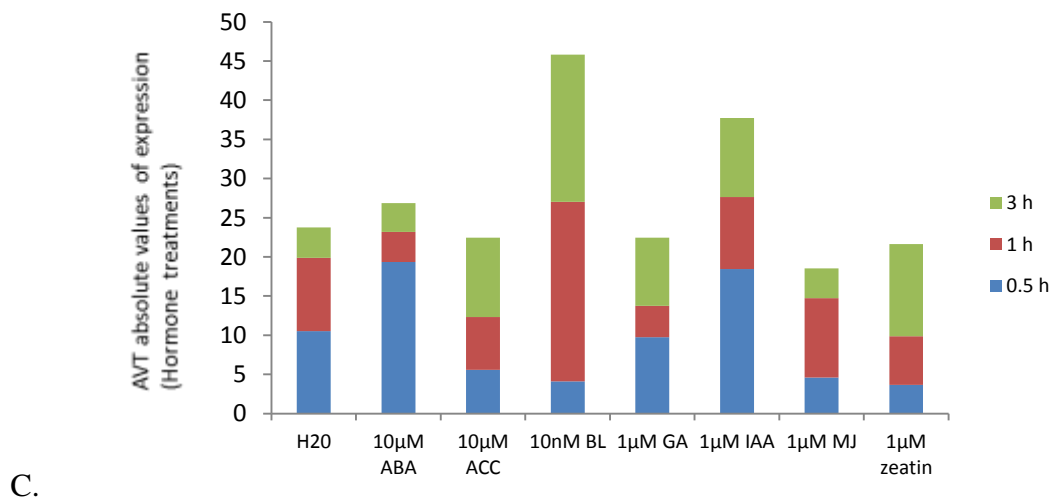
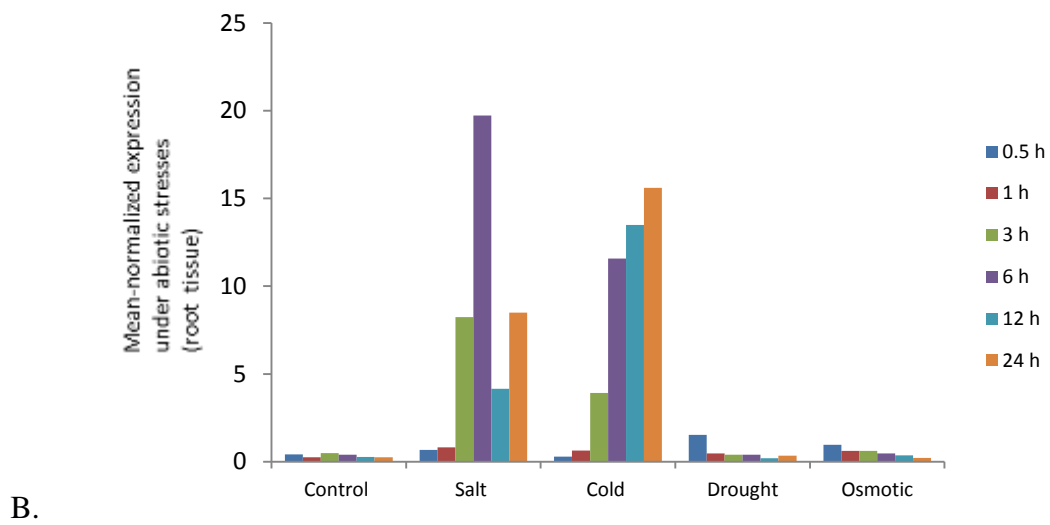
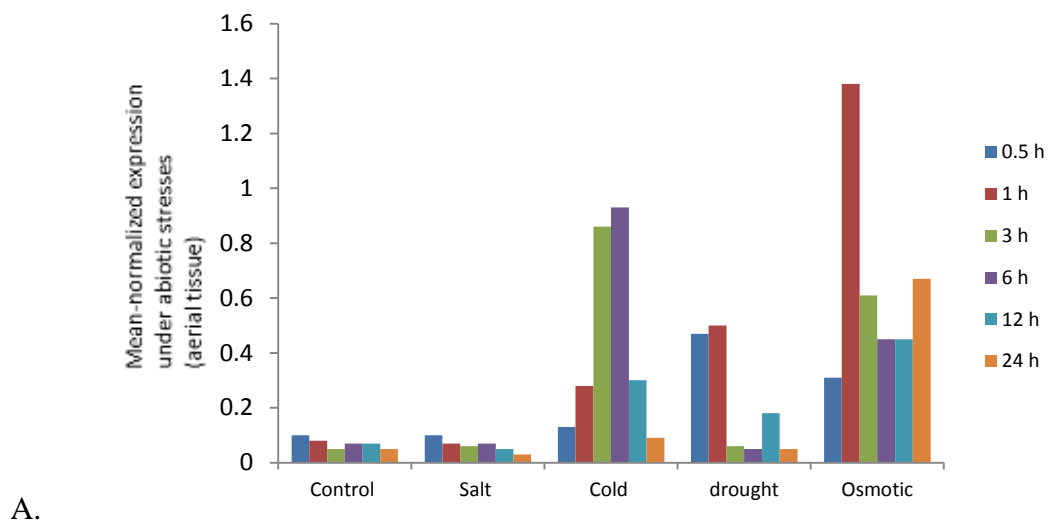
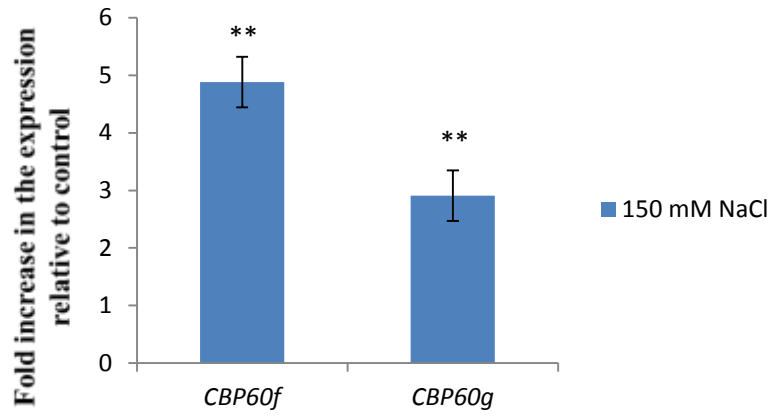
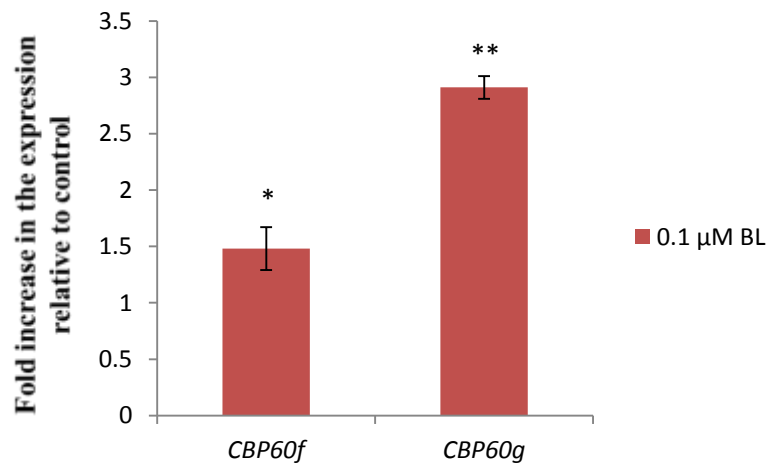


Figure 3.3: Salt stress and BR responsive expression of *CBP60g* and *CBP60f*

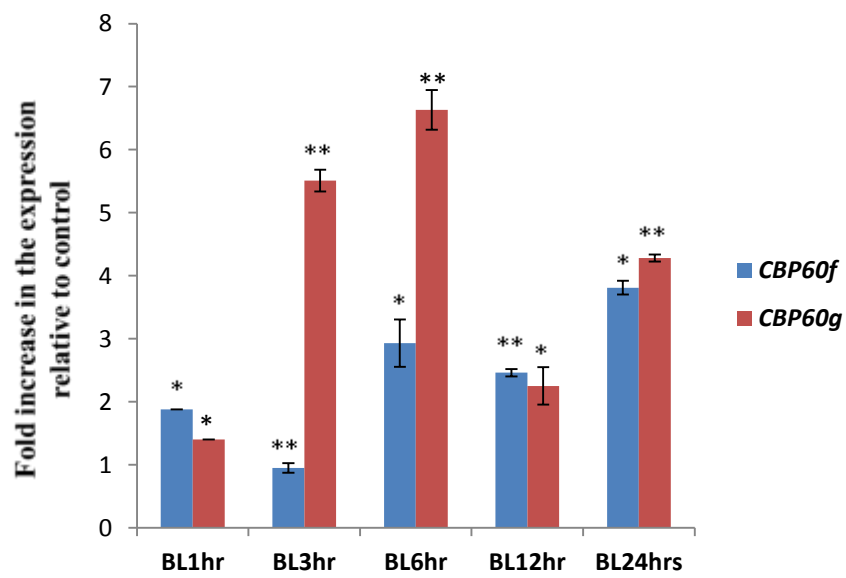
- A) Salt-induced upregulation of *CBP60g* and *CBP60f* expression. WT *A. thaliana* seedlings grown on soil for 21 days were treated with 150 mM NaCl for two weeks. Total RNA isolated from leaf tissue of these plants was used for qRT-PCR, n=3 and Student's t-test (* $P < 0.05$, ** $P < 0.01$).
- B) BR-induced *CBP60g* and *CBP60f* expression following long-term treatment. Leaf tissue of WT *A. thaliana* seedlings grown for 21 days on MS medium containing 0.1 μ M BL was used for RNA extraction followed by qRT-PCR, n=3 and Student's t-test (* $P < 0.05$, ** $P < 0.01$).
- C) BR-induced *CBP60g* and *CBP60f* expression following short-term treatment. Leaves from 21-day-old WT *A. thaliana* seedlings were floated on liquid MS medium containing 0.1 μ M BL for 1, 3, 6, 12, and 24hr. Total RNA extracted from these samples was analyzed by qRT-PCR. One-way ANOVA was used for statistical analysis (* $P < 0.01$, ** $P < 0.001$).



A.



B.



C.

transcript was 1.8, 0.9, 2.9, 2.4 and 3.8 -fold higher in BR-treated leaf samples as compared with untreated controls at 1h, 3h, 6h, 12h and 24hr, respectively. Similarly, *CBP60g* expression was increased 1.4, 5.5, 6.6, 2.2, and 4.28 -folds at 1h, 3h, 6h, 12h and 24hr of treatment, respectively (Figure 3.3C). These data confirmed that *CBP60g* and *CBP60f* are BR and salt stress -induced genes.

3.2 Sequence analysis of *CBP60f* and *CBP60g*

CBP60f encodes a 562 amino acid protein with a predicted molecular mass of 63.7 kDa, whereas *CBP60g* encodes a 563 amino acid protein with 63.08 kDa molecular mass. A search in the NCBI's Conserved Domain Database identified the presence of a Calmodulin Binding Domain (CaMBD) between aminoacid residues 91 to 388 in *CBP60f* and residues 85 to 371 in *CBP60g*. Most of the *CBP60* proteins have CaM-binding site at C-terminus end, whereas *CBP60g* showed the presence of CaM-binding site towards N-terminus end (Wang et al., 2009) (Figure 3.4).

Multiple sequence alignment of *CBP60* protein members showed a highly conserved central region with sequences that were more than 90% similar to one another. (Figure 3.4). *CBP60f* protein was predicted to be localized in the cytoplasm using PSORT and SubLocv1.0 tools, while *CBP60g* was predicted to be localized to the nucleus. The nuclear localization of *CBP60g* was confirmed in a previous study (Wan et al., 2012).

The promoter area 1,500 bp upstream from transcriptional start site of each gene was analysed by Signal Scan Search software using PLACE database (<http://www.dna.affrc.go.jp/PLACE/signalscan.html>) and PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). Motifs involved in light responsiveness such as the GATA box, W box and ACE motifs (Jeong and Shih, 2002; Luo et al., 2010) were identified in both gene promoters. One P-box (responsive to gibberellins) and two TGACG motifs (responsive to jasmonate) were found in the putative *CBP60f* promoter. Various motifs involved in stress responsiveness such as MBS (MYB binding motif), HSE (heat stress responsive element), circadian motif, ERE (Ethylene responsive element), and TC rich motifs were present in promoter regions of *CBP60g* and *CBP60f*. The promoter regions of *CBP60g* also showed the DNA binding

Figure 3.4: Multiple sequence alignment of Arabidopsis CBP60 proteins. Coding sequences of Arabidopsis CBP60 proteins were aligned using Multalin with default settings (<http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html>). Red colored amino acids: consensus value>90%, blue colored amino acids: consensus value>50%. Purple underlined region indicates the experimentally determined calmodulin binding domain (CBD) of CBP60g, red colour underlined region indicates conserved CBDs of CBP60a, CBP60b, CBP60c, CBP60d, and CBP60e.

	1	10	20	30	40	50	60	70	80	90	100	110	120	130	
CBP60a	NRIPYDFGSKFSVYQVYRLQTVKHFLEPYLEPLIRKVVYKEVELALGK---HLAGIKVICEKETHPLESRN-LQLKFLNNLSLPVFTSRIEAGEGQAIRV														
CBP60b	MNDGNNNNHNRKRNLDGNDODK---PERKRPALASVIVEALKVDSQLQLCSS-LEPLIRVVYSEELERALKLGPRLTGSSGSSPKRIEGPDGAK-LQLHFKSRLSLPLFTGGKVEGEQGAIVH														
CBP60d	MKRNFERNDDK---PERKRPALASVIVEALKVDSQLQLCSS-LEPLIRVVYSEELERALKLGPRLTSSVSPKRIEGPDGAK-LQLHFKSRLSLPLFTGGKVEGEQGAIVH														
CBP60e	HQTRYHRTNSHREKRLLEDONQQQQQPERKRPALASVIVEALKVDSQLQLCSS-LEPLIRVVYSEELERALKLGPRLSERS--SPKRIEGTGGN-LQLQFASRLSVPLFTGGKIEGEQGAIVH														
CBP60f	MNRKGYECSQEDTKLPESKRQKVPALASVIVEALKVDSQLQLCSS-LEPLIRVVYSEELERALKLGPRLTSRSP-EPKRIQDRNGN-LQLHFRTRNPPHPLFTGGKVEGEQGAIVH														
CBP60g	MENSNNHNGHGNQENADNLPESKRQKVPALASVIVEALKVDSQLQLCSS-LEPLIRVVYSEELERALKLGPRLTSRST-EPKRIQDLGNGN-LQLHFRTRNPPHPLFTGGKVEGEQGAIVH														
CBP60h	MKIRNSPSFHGGSGYSVFRARMLTFKKYVKKYHDSNNQHTD-HENIRIRVREIQRSLQPLFSSSCYSH---ERSRSETSPSSRSRLKCFINSPSSITFGSKIEADGSPLYI														
CBP60h	MAGKRLFDQLDSQENKSEKRIKSYVPSLSPSSVFGALISENTLSVLEPVIRKVRQVEYVIGSKRFLSRSSSFRIEPEATTP---LKLTFRKHLNTPITFGSKISDYDNNMPLI														
Consensusp.lasv!.ev.....s.....s.#Ep.iRr!Y.#E!#al.k.....l.s.....e.kr...p..rn.lq.L.F...l..p.Ffg.k!ege.g..i.!.														
	131	140	150	160	170	180	190	200	210	220	230	240	250	260	
CBP60a	GLIDPSTGQIFSSGPASSAKLEVYVEGDFNS---VSDHTDEDIRNMYREREGKPLLNQVFAVNLNIGVGN-DEISFTDNSSHTSRKFLGVRIYDQF---DYVKIREAITESFYVKDRGELYKKH														
CBP60b	VLIDANTGRAYVYGPERSAKLHIVYLEGDFNDEDDHTDEEFESHVYKERSGKRPLLTGEVYVTLKEGVTL-GEIVFTDNSSHTSRKFLGLRVYSGC-CDGMRIRKATEFVYKDRGELYKKH														
CBP60d	VLIDANTGRPYVYGPERSAKLEVYVLEGDFNDEDDHTDEEFESHVYKERSGKRPLLTGLVYVTLKEGVTL-GEIVFTDNSSHTSRKFLGLRVPSGY-CDGIRIRKATEFVYKDRGELYKKH														
CBP60e	VLLDHTTGHLTVYGPERSAKLEVYVLEGDFNDEDDHTDEEFESHVYKERSGKRPLLTGDVYVTLKEGVTL-GEIVFTDNSSHTSRKFLGLRVPSGY-CEGMRIRKATEFVYKDRGELYKKH														
CBP60f	VLIDANTGNVYVQTEESAKLHVVYLEGDFNDEDDHTDEEFESHVYKERSGKRPLLTGDVYVTLKEGVTL-GEIVFTDNSSHTSRKFLGLRVPSGY-GDSFCIRKATEFVYKDRGELYKKH														
CBP60g	VLIDANTGNVYVQTEESAKLHVVYLEGDFNDEDDHTDEEFESHVYKERSGKRPLLTGDVYVTLKEGVTL-GEIVFTDNSSHTSRKFLGLRVPSGY-FHDIRKATEFVYKDRGELYKKH														
CBP60h	ELVDATTNTLVSTGPFSSSRVLYPLNADFT---EESMTVEGFNRNLTQREGKRPLLTGLVYVTLKEGVTL-GEIVFTDNSSHTSRKFLGLRVPSGY-GDGVEARSEAFGCRDQGESYKHH														
CBP60h	ILYDQSNKPYNLNRP---IXLQIVYALHGDFPS---GDKVTSDEEFSNLIKERDGRKPLLAGVSYVTVRNGVATL-GEIVFTDNSSHTSRKFLGAKVAKGSSGQGVYVCEMTAIVYKDRGELYKKH														
Consensus	.LiDa.tg.....gp.ss.kl#.vvl.gDfn.....dR.#.feanivk#*GKrPillGtv.v.lk#G!gt..gei.FDNSSHTSRKFLG.k...g....g..irEA.tEaf.vrDRGELYKKH														
	261	270	280	290	300	310	320	330	340	350	360	370	380	390	
CBP60a	PPSLFDEVHRLKIKGDFAFHKLNLGNINTYKDFLTHFHLNSSLRQVLGTGHSKNAEITLDHARSCLVSSVHYVQAPGQKTAIVFVYVQVLLVDFQYIPREKLSIEKQAREVYVDRKSH														
CBP60b	PPALNDVHRLKIKGDFAFHKLTAEGINTYDFLRVYVQSPKRLTILGSGHSMKADALVEHAKTCVQSSKLYIYYAD-SRNVGVVFNMIYELSLISGQYVSAISLTSQKVVYVGLYKRAYEN														
CBP60d	PPALNDVHRLKIKGDFAFHKLTAEGINTYDFLRVYVQSPKRLTILGSGHSMKADALVEHAKTCVQSSKLYIYYAD-SRNVGVVFNMIYELSLITEDQYVSAISLTSQKVVYVGLYKRAYEN														
CBP60e	PPALNDVHRLKIKGDFAFHKLTAEGINTYDFLRVYVQSPKRLTILGSGHSMKADALVEHAKTCVQSSKLYIYYAD-SRNVGVVFNMIYELSLITEDQYVSAISLTSQKVVYVGLYKRAYEN														
CBP60f	PPALNDVHRLKIKGDFAFHKLTAEGINTYDFLRVYVQSPKRLTILGSGHSMKADALVEHAKTCVQSSKLYIYYAD-SRNVGVVFNMIYELSLITEDQYVSAISLTSQKVVYVGLYKRAYEN														
CBP60g	PPALNDVHRLKIKGDFAFHKLTAEGINTYDFLRVYVQSPKRLTILGSGHSMKADALVEHAKTCVQSSKLYIYYAD-SRNVGVVFNMIYELSLITEDQYVSAISLTSQKVVYVGLYKRAYEN														
CBP60h	PPALNDVHRLKIKGDFAFHKLTAEGINTYDFLRVYVQSPKRLTILGSGHSMKADALVEHAKTCVQSSKLYIYYAD-SRNVGVVFNMIYELSLITEDQYVSAISLTSQKVVYVGLYKRAYEN														
Consensus	PP.LI.DEVHRL.#kIGDgafhklk...I.t.V.#Frl.v.#.klr.iig.6s.kw#.tv.Ha..Cil..kly.gy.....vvfN.!g#.gl...g#.s.#.l...#.d.#v.#g.n														
	391	400	410	420	430	440	450	460	470	480	490	500	510	520	
CBP60a	LNEYISYDDEVSMRMYLNAPASQGSVAGIDYSLTSLDGYGYFSSLLNTRECSGKHSDDVMEVTPHGLYEDYNLWNCNHLGLEEPOSELQSLDDFHSQKASVGGKASKRAKTLFSYSRALS														
CBP60b	HNLVIEYDGG-SLLDLQPKRLSITHNLNEMYSTARIDHPHMYAGHSSHPNPQSPVLSDFAGIGYDQTLATRYHSHPQLLNSNPRQFEVASCSTSQDQFNG-NLHQTQSTINQHNLGLGPSQSS														
CBP60d	HNLVIEYDGG-SLLNLNQLPERLDISQD-----PYTALASYST-VPLSQF-----EFAIEGYNTLTTA-----LPHNQAFQDFVP-----QQQFQIGTQQTQNTIENHYTRLVLGPSS														
CBP60e	HNLVIEYDGG-SLLHNFNQVSKTD-----DIDYSPHVSVPQSTSYSDVTEAYNQSPASSFPQSQ-LADTTYHAFGNSSFA-PQDQLVN-NTHESQSHINSNGGYRLGAPATGS														
CBP60f	HNLVIEYDGG-SLLNCLPVAKKIKSL-----LEPKHVSQATAPNAQHLHQNQATYQGHQNAITYSPYQPIDYQFAQHCNQLLPSPCKVQDYNRSHSSSSSYNGEDACPPRAH														
CBP60g	HNLVIEYDGG-SLLNCLPVAKKIKSL-----LEPKHVSQATAPNAQHLHQNQATYQGHQNAITYSPYQPIDYQFAQHCNQLLPSPCKVQDYNRSHSSSSSYNGEDACPPRAH														
CBP60h	HNLVIEYDGG-SLLNCLPVAKKIKSL-----LEPKHVSQATAPNAQHLHQNQATYQGHQNAITYSPYQPIDYQFAQHCNQLLPSPCKVQDYNRSHSSSSSYNGEDACPPRAH														
Consensus	.n.v..y.....s.....l.....														
	521	530	540	550	560	570	580	590	600	610	620	630	640	650	
CBP60a	VFKYVYKGI														
CBP60b	TSGYQNIIMPSSVHQADLNHLEDHNSPRERGPDFFSEEEIRLSHMELESDHQQLRLFSHGG---GGNGSATHLPEDGYTFPSFL-HTPM-QGYDED-RGSGRAYVYVGLKIKARHAGFFIRKARE														
CBP60d	TGGYQDIK-SSAQENLNPFEDHNLSE---NDFFSEEEIRQTSHDLANEHQQL---FSHGG---GKG---EDGFTFSPHNTPHMGYDEEGRGSGRAYVYVGLKIKARHAGFFIRKARE														
CBP60e	QNGEQLVHPP---EINSYNDHSMTCNRGVDGFLSEEEIRARSHMELENDHQQLRLFSHGG---GKG---EDGFTFSPHNTPHMGYDEEGRGSGRAYVYVGLKIKARHAGFFIRKARE														
CBP60f	GQGLDI-----FSEIRLSHMELETDHQQLRLKTFGTYNTVGTGGFGQDSCYGY-SIPYQADINTYRERNRSGRAYVYVGLKIKARHAGFFIRKARE														
CBP60g	GQGLDI-----FSEIRLSHMELETDHQQLRLKTFGTYNTVGTGGFGQDSCYGY-SIPYQADINTYRERNRSGRAYVYVGLKIKARHAGFFIRKARE														
CBP60h	TYYTKGC-----IENNEEDENAFSYHNDHTSSH-----SPGTHQAVETHFLTYSETEEAGFVDVFNANVNLGSPRA-RUCKYKRAFKYRAAFKEVRRH														
Consensuse.....dn.....e.....s.....s..a...u.k.kaa.....														
	651	660	666												
CBP60a	RRRAQIVELDDDDDEGE														
CBP60b	RRRAQIVELHNDGK														
CBP60d	RRRAQIVQLDEDE														
CBP60e	RRPQIVEID														
CBP60f	RRPQIVEID														
CBP60g	TTRNPREGL														
CBP60h															
Consensus															

Figure 3.5: Promoter sequence analysis. The upstream sequences of CBP60f and CBP60g showing 1500 bp of promoter (depicted in black letters). The positions of motifs for E-box, MBS motif, HSE, Pbox, circadian, TGAGC motif, ACE motif, GATA box, W box, TC rich repeats, are highlighted in yellow, grey, dark green, blue, red, sky-blue, purple, pink, light green and light grey. ERE motif and DNA binding AT rich region in *CBP60g* promoter sequence are mentioned in orange and red coloured letters respectively. **ATG** represents the translational start site.

A) 1500 bp upstream sequence for *CBP60f*, highlighted regions with specific colour indicates a motif.

B) 1500 bp upstream sequence for *CBP60g*, highlighted regions with specific colour indicates a motif.

CATCTG- E-box

GTTATT- MBS motif

CGTCA- TGAGC motif

CAAAAGG- P-box

TCCACGTT

TCCTAT

CAAGAGGATC- Circadian

CGTTT

ATCTTCCTG –Heat shock motif

TTAATTCTAT

ATTTCAAA

ATG- translational start site

Maximum upstream distance: 1500

Maximum upstream distance: 1500

44

AT rich region. A manual search of these regions identified seven E box elements (CANNTG), the binding site for transcription factor BES1, in *CBP60f* and three E-box elements (CANNTG) in *CBP60g* promoter (Figure 3.5A and B). The enriched presence of E-box elements in the putative promoter regions is evidence in support of the notion that *CBP60f* and *CBP60g* are BR-regulated genes.

3.3 CBP60f is localized to the nucleus

According to PSORT and SubLocv1.0 tools used to predict the localization of proteins, CBP60f should be localized to the cytoplasm. To check this, the yellow fluorescent protein (YFP) was fused separately to the N- and C- terminus of full-length CBP60f and the fusion protein was transiently expressed in leaf tissue of *N. benthamiana*. Under microscopic examination, the YFP-CBP60f fusions were localized to both the cytoplasm and the nucleus (Figure 3.6A). DAPI staining was used to confirm the nuclear localization of CBP60f (Figure 3.6B). Nearly more than 35% of total stained CBP60f protein appeared to be localized in nucleus when quantified manually.

3.4 Knockout mutations in CBP60g and CBP60f lead to salt-sensitivity

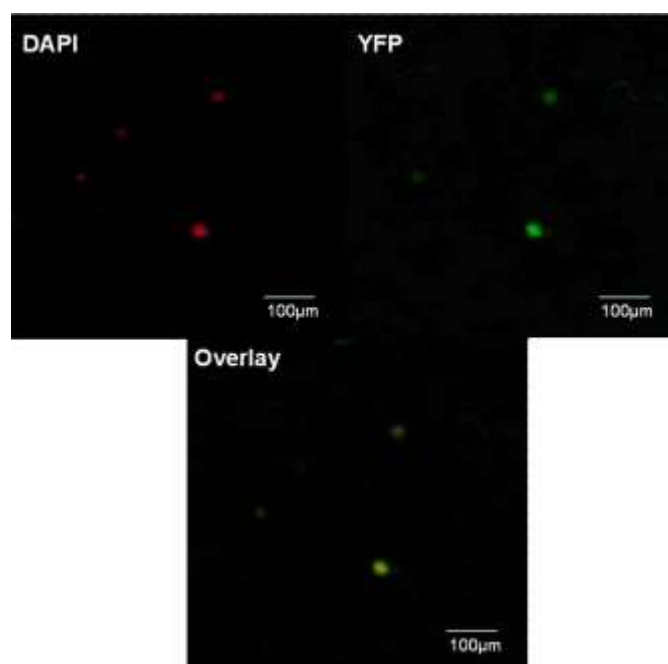
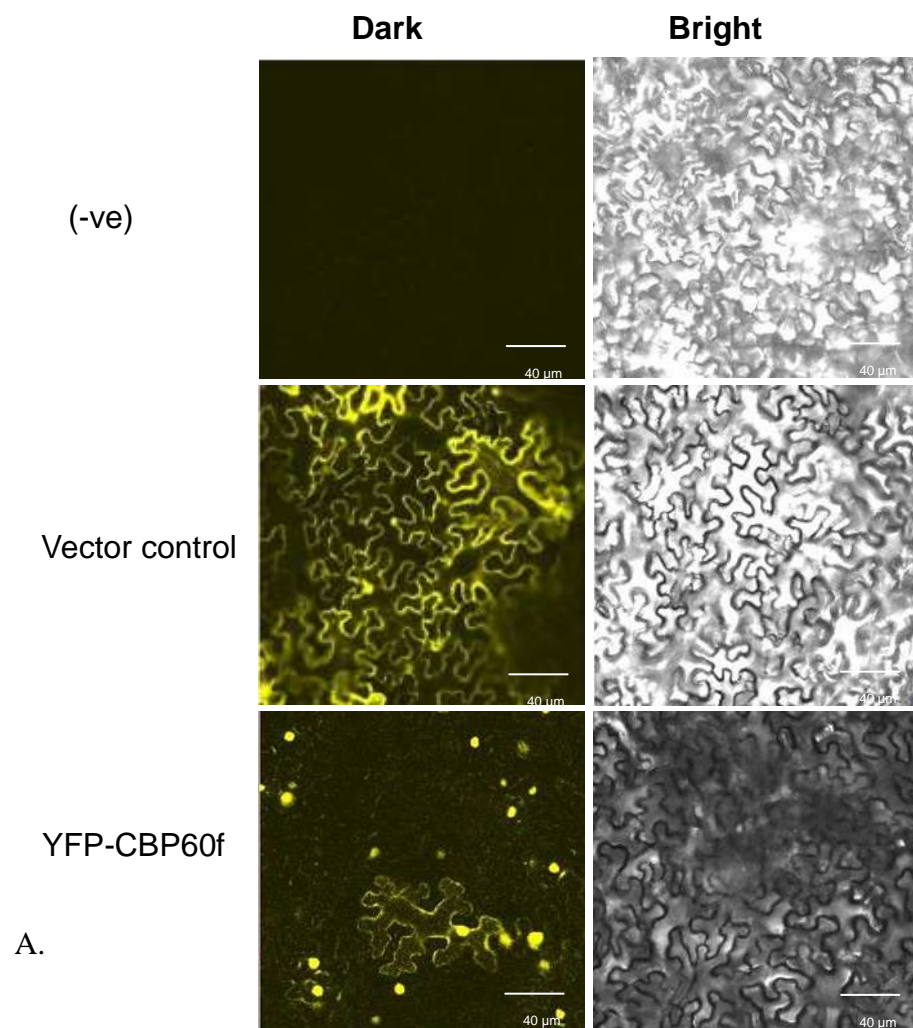
A reverse genetic approach was used to analyse the role of *CBP60g* and *CBP60f* in salt stress responses of *A. thaliana*. Confirmed homozygous T-DNA insertion mutants, *SALK_023199c* and *CS860821* for *CBP60g* and *CS860840* for *CBP60f* were obtained from ABRC. In all 3 cases, T-DNA insertions were present in the exon regions (Figure 3.7A). RT-PCR analysis performed using gene specific primers confirmed that transcript expression from these genes was present in WT but completely abolished in the mutants (Figure 3.7B). Hereafter, mutants *SALK_023199c* and *CS86082* are referred to as *cbp60g-1* and *cbp60g-2*, respectively, and *CS860840* as *cbp60f*.

It is well established that seed germination and normal seedling growth are inhibited under high salt conditions (Zhu et al., 2005). To check the effect of salt stress on germination, the mutants and WT seeds were germinated on MS medium containing different concentrations of salt (0 mM, 10 mM, 150 mM, or 200 mM NaCl). While all genotypes showed equal germination in the absence of salt, the germination percentages

Figure 3.6: Subcellular localization of CBP60f

A) Images by confocal microscopy showing the localization of the YFP-CBP60f fusion construct. YFP-CBP60f fusion construct was transiently expressed in *N. benthamiana* leaf tissues under the control of the cauliflower mosaic virus 35S promoter, and the epidermal cells were examined through confocal microscope. The photographs were taken in the bright field for the morphology of the cells, in the dark field for yellow fluorescence.

B) Images by confocal microscopy showing the nuclear localization of CBP60f. The photographs show the DAPI stained nucleus and presence of YFP-CBP60f fusion protein in nucleus confirmed by overlapping both of them.



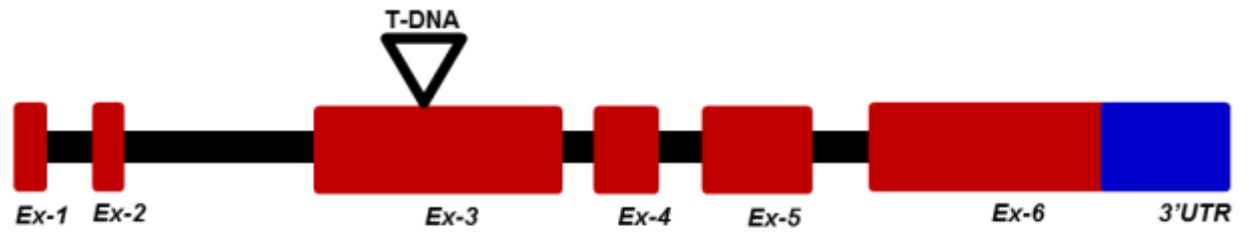
B.

Figure 3.7: T-DNA insertion mutants and RT-PCR analysis

A) Schematic diagram showing the T-DNA insertion site in the *cbp60g* mutants (Salk_023199c- *cbp60g-1*, CS860821- *cbp60g2*) and *cbp60f* mutant (CS860840). The organization of introns, exons, and untranslated regions (UTR) of the *A. thaliana* *CBP60g* and *CBP60f* genes are depicted by lines, and red and blue-colored boxes, respectively. The T-DNA insertion site is indicated by downward triangle.

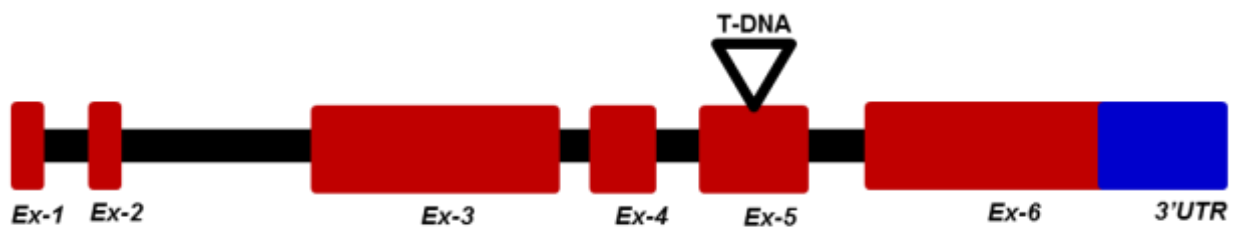
B) Analysis of *CBP60g* and *CBP60f* mRNA expression in WT and mutant seedlings by RT-PCR using gene specific primers. RNA was isolated from 21-dayold WT and mutant plants grown on soil. *ACTIN* was used as an internal control.

A.



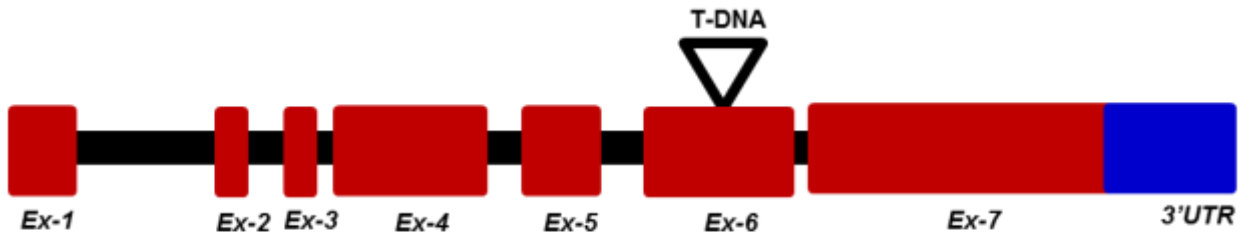
Salk_023199c

cbp60g-1



CS860821

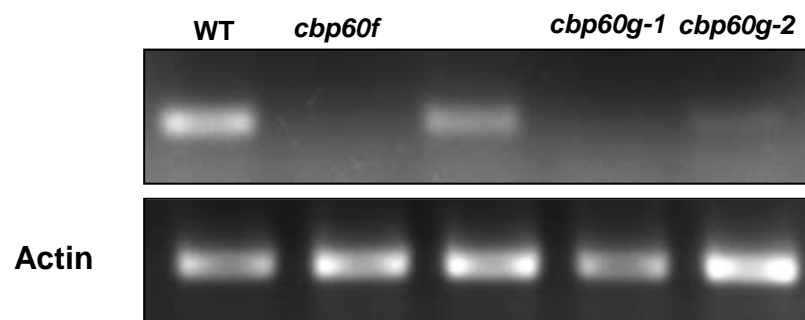
cbp60g-2



CS860840

cbp60f

B.



were increasingly inhibited by increasing salt concentrations. Germination of *cbp60f* seeds was much more sensitive to salt as compared to WT and *cbp60g* (Figure 3.8). To further explore the role of *CBP60g* and *f* in salt stress tolerance, 3 weeks old WT, *cbp60g-1*, *cbp60g-2* and *cbp60f* seedlings grown on soil were subjected to salt stress by watering plants with 150 mM NaCl solution for three weeks. After 2 weeks of salt treatment, mutant plants started to turn yellow and senesce, which was earlier and to a greater extent than WT (Figure 3.9A and B). At the end of three weeks, only 25% of *cbp60g-1*, 31% of *cbp60g-2*, and 6% of *cbp60f* plants survived. By comparison 60% of WT plants survived the treatment (Figure 3.9C). These data together with the salt stress-induced upregulation of gene expression suggest that both *CBP60g* and *f* have roles in salt tolerance mechanism of the Arabidopsis plant.

3.5 Overexpression of CBP60f in transgenic plants leads to enhanced salt stress tolerance

In order to elucidate the role of *CBP60f* in salt stress tolerance, *CBP60f* full-length cDNA was expressed under the control of CaMV 35S promoter. From the 20 independent transgenic lines that were isolated initially, six OE lines were selected for further analysis based on 100% survival against BASTA treatment. Preliminary examination of *CBP60f* overexpressing (OE) lines appeared to have a thick stem and broad leaf phenotype compared to mutant and WT. qRT-PCR analysis of *CBP60f* expression in shoot tissue of 14-day-old seedlings showed that *CBP60f-1*, *CBP60f-2*, *CBP60f-3*, *CBP60f-4*, *CBP60f-5* and *CBP60f-6* had 6.2, 5, 7, 9.5, 3.3, 6.1 –fold higher expression, respectively, than WT (Figure 3.10B). When subjected to salt stress treatment (as described in section 2.3), all transgenic lines showed improved salt tolerance than WT and *cbp60f* within the first 15 days of treatment. Following three weeks of salt stress (Figure 3.10A) the transgenic lines had survival rates ranging from 68-84% while only 48% of WT plants survived (Figure 3.10C). Only 4% of the *cbp60f* plants survived after three weeks of salt stress treatment.

Figure 3.8: Germination pattern of mutants under different salt concentrations.

A) *WT*, *cbp60f*, *cbp60g-1* and *cbp60g-2* seedlings were allowed to germinate on MS plates containing different concentrations of NaCl (0 mM, 100 mM, 150 mM, and 200 mM NaCl) and observed for cotyledon emergence. The photographs were taken after 5 days.

B) Proportion for germination percentage for WT and mutants at different concentrations of NaCl. All the experiments were repeated three times and vertical bars represent Standard Error (SE) of mean for three replicates.

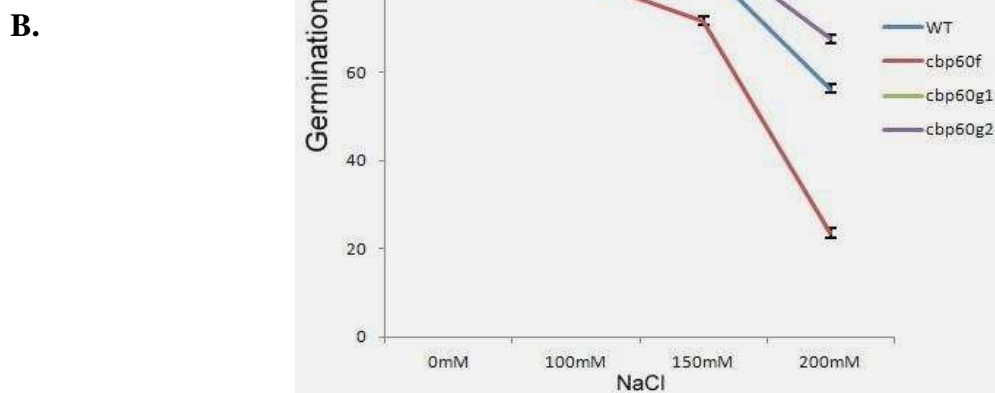
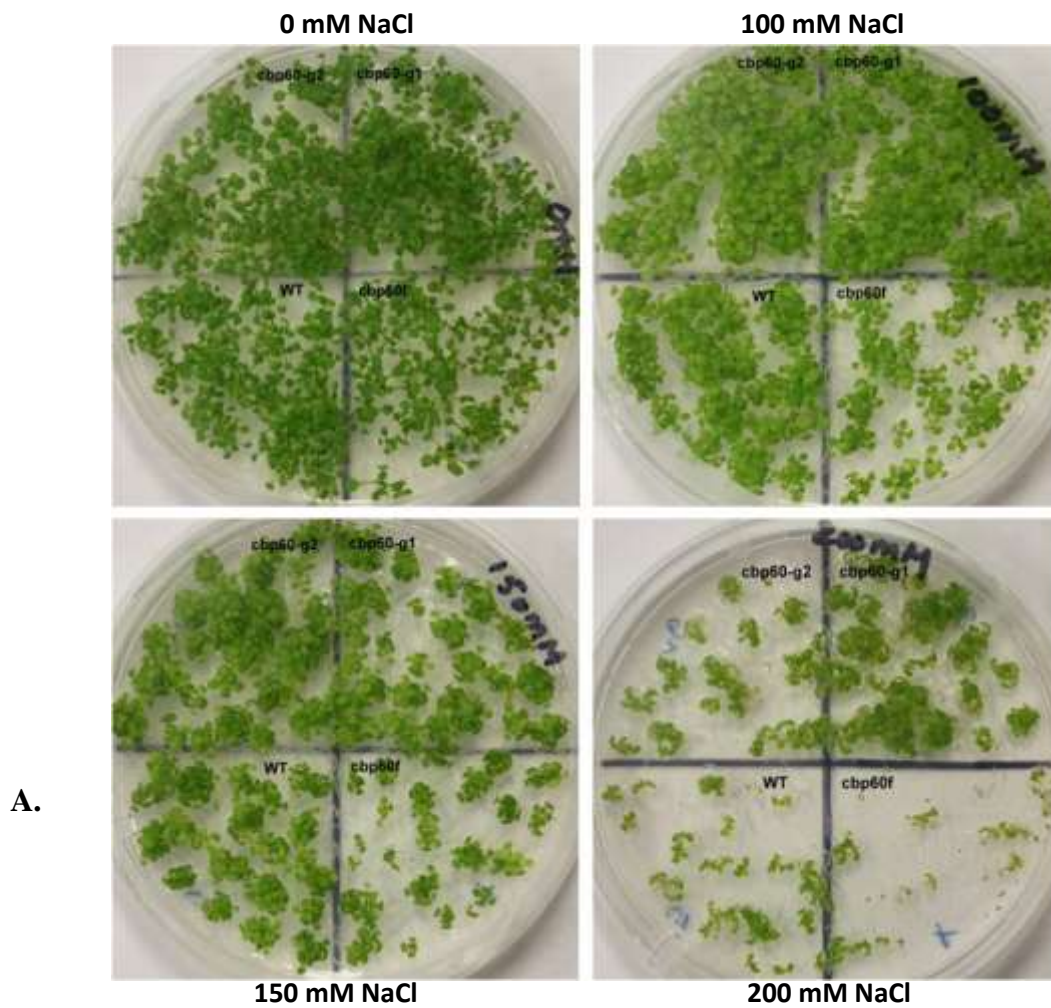
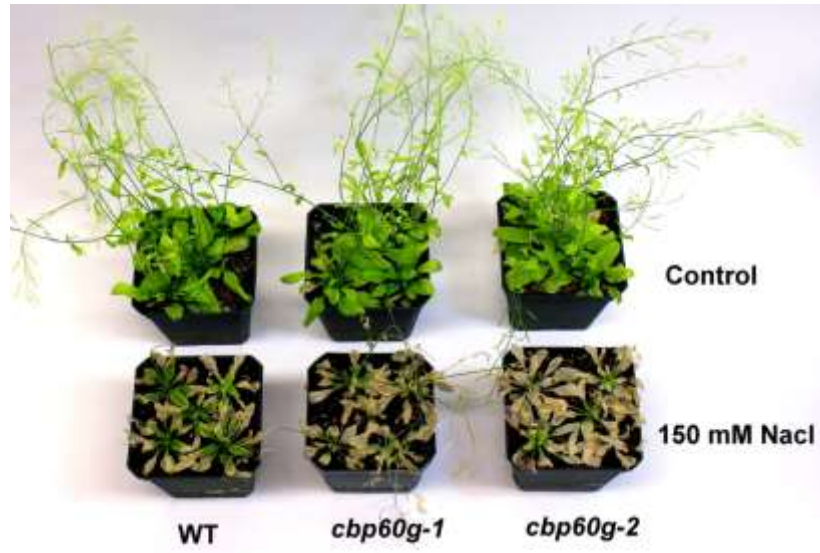


Figure 3.9: Mutation in *CBP60g* and *CBP60f* lead to salt sensitivity

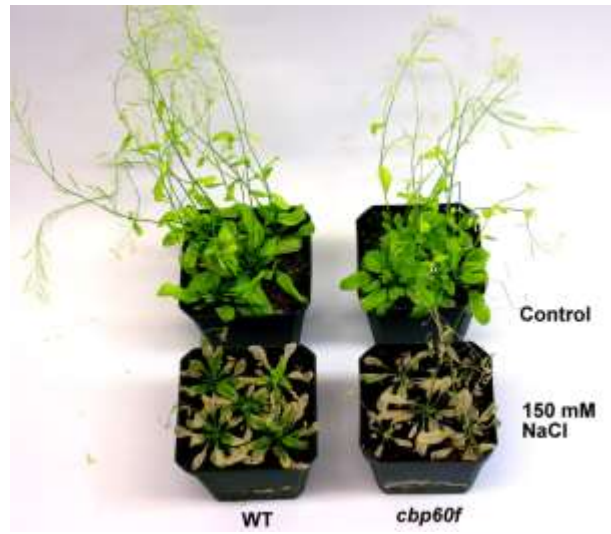
A) WT and *cbp60g* mutants were grown on soil for three weeks and subjected to salinity stress treatment (watering every two days with water containing 150 mM NaCl). Photographs of the control (mock-treated) and salt- stressed plants were taken after 25 days of salt treatment.

B) WT and *cbp60f* mutants were grown on soil for three weeks and subjected to salinity stress treatment (watering every two days with water containing 150 mM NaCl). Photographs of the control (mock-treated) and salt- stressed plants were taken after 25 days of salt treatment.

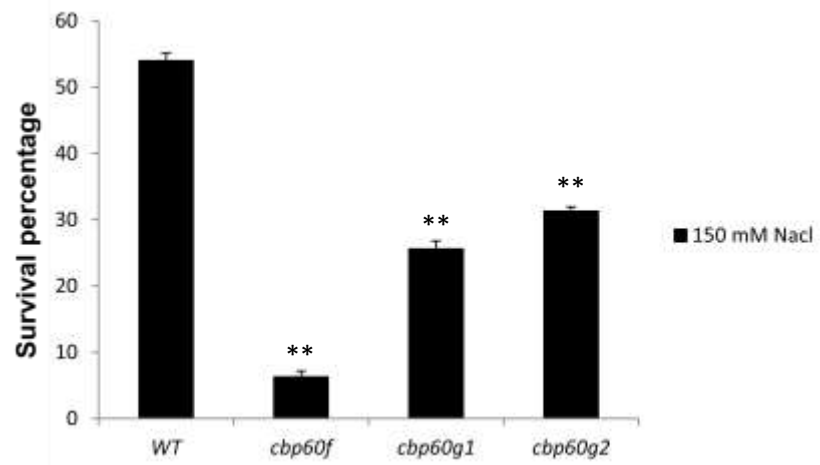
C) Plot showing the survival percentages for WT and mutants after three weeks of salt treatment. All the experiments were repeated three times and vertical bars represent Standard Error (SE) of mean for three replicates. Student's t-test was done, $n=3$ (** $P<0.01$).



A.



B.



C.

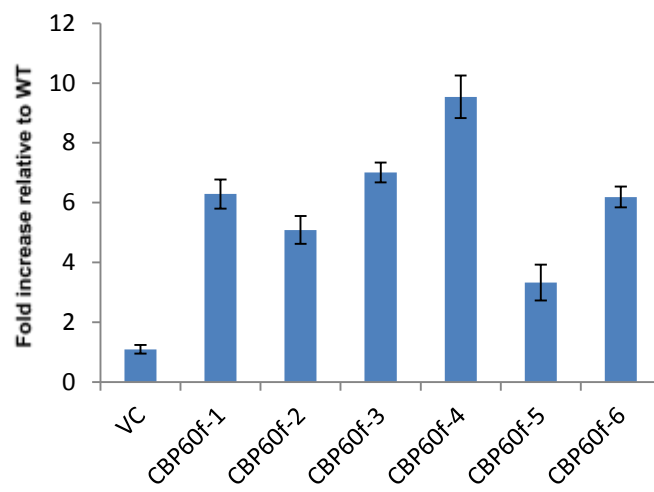
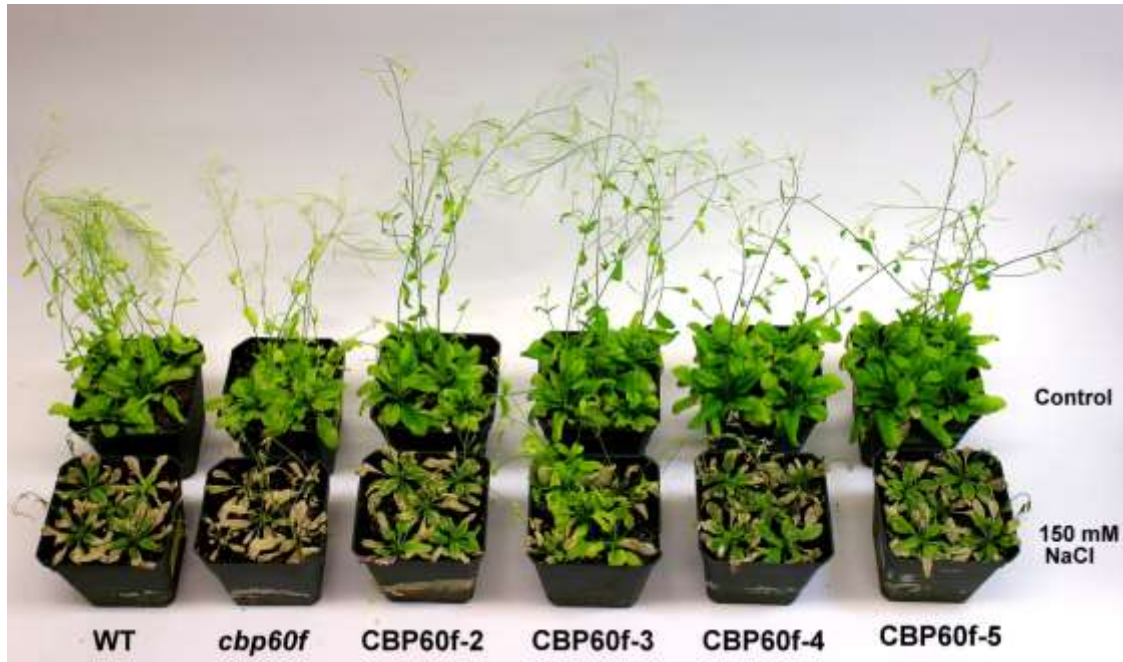
Figure 3.10: *CBP60f* overexpressing plants are more tolerant to salt stress than WT and *cbp60f*.

A) WT, *cbp60f*, and *CBP60f* overexpressing lines (CBP60f-2, CBP60f-3, CBP60f-4 and CBP60f-5) were grown for 3 weeks on soil and subjected to salt stress (watering every two days with water containing 150 mM NaCl). The photographs of control (mock-treated) and salt-stressed plants were taken after 25 days of salt treatment.

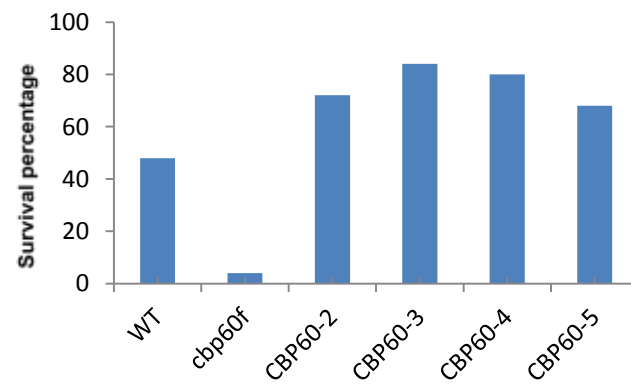
B) Real time PCR analysis of *CBP60f* transcript in WT, vector control and overexpressing lines. The data is expressed in fold increase compared to WT. Standard error for the mean of three biological replicates was mentioned as small vertical bars.

C) Plot showing the survival percentages for WT and OE lines after three weeks of salt treatment.

A.



B.



C.

3.6 Overexpression of CBP60f improves root length during salt stress

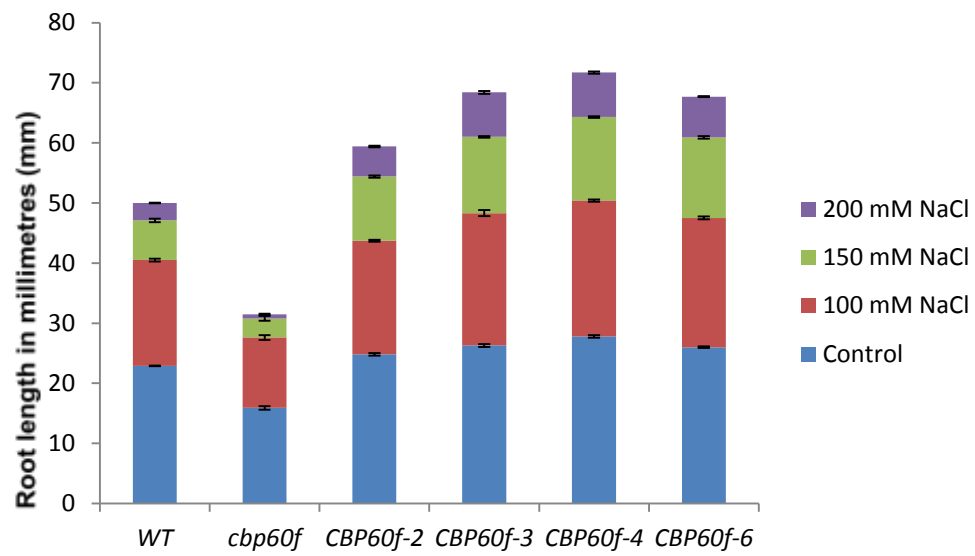
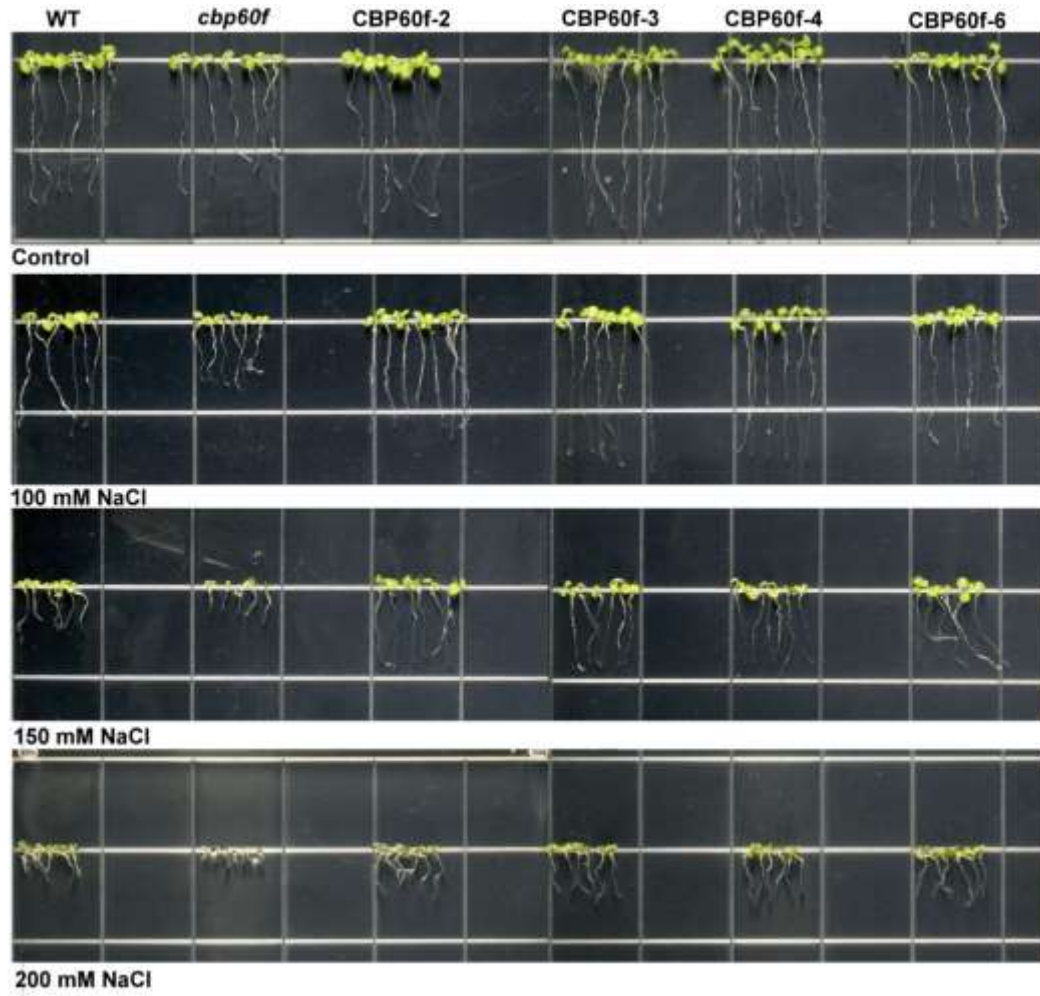
Since *cbp60f* mutant growth and seed germination was highly inhibited under high salt condition, we also checked the behavior of roots under salt and normal conditions compared with WT and *CBP60f* OE lines. Seeds for WT, *cbp60f* and *CBP60f* OE lines were allowed to germinate on MS medium plates containing different concentrations of NaCl (0 mM, 100 mM, 150 mM or 200 mM NaCl) and incubated vertically in growth chamber. All the OE lines showed the increase in root length compared to WT and the *cbp60f* mutant under salt stress (Figure 3.11). The root length of all the seedlings were measured using a standard ruler and compared with WT. Interestingly, all the *CBP60f* OE lines showed increased root length phenotype compared to WT under normal conditions (Figure 3.12).

Figure 3.11: Root phenotypes of WT, *cbp60f* and OE lines under different concentrations of salt

A) WT, *cbp60f*, and CBP60f overexpression seedlings were allowed to germinate vertically on MS medium containing different concentrations of salt (control, 100 mM, 150 mM, or 200 mM NaCl). All the pictures were taken after one week of germination.

B) Root lengths for above mentioned experiment was measured and plotted as a bar graph.

A.



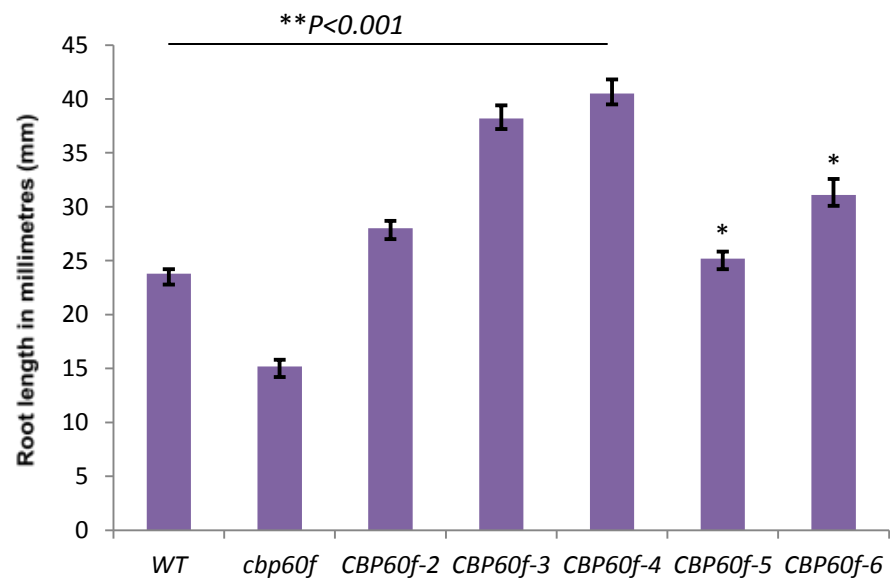
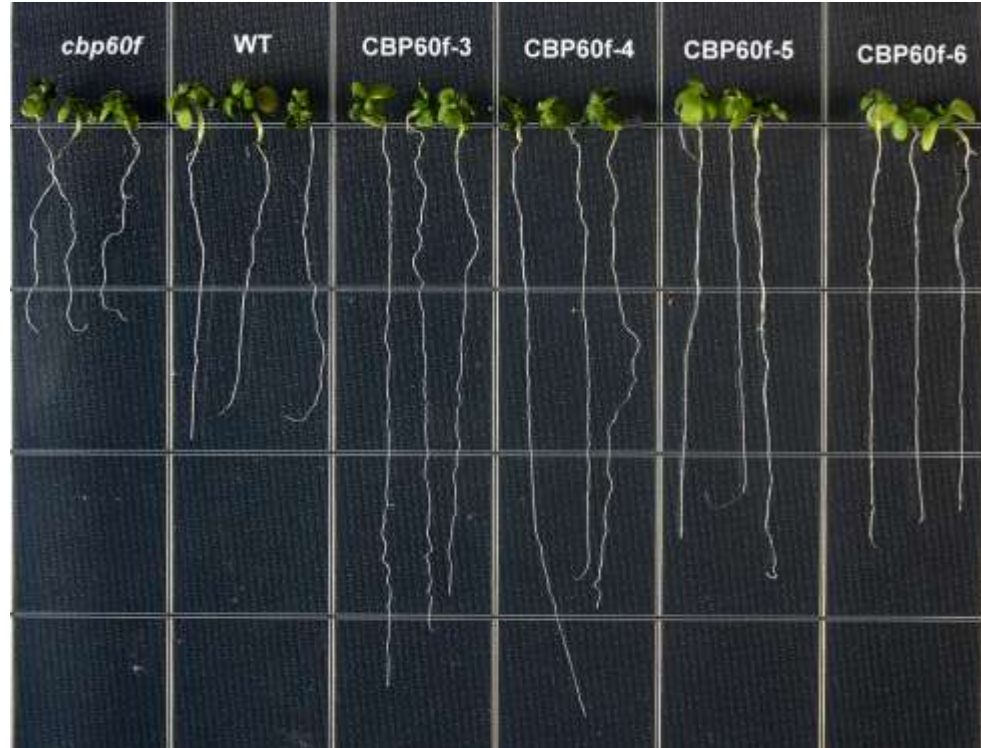
B.

Figure 3.12: CBP60f overexpression lines showing increased root length compared to WT and *cbp60f*.

A) WT, *cbp60f* and OE line seedlings were allowed to germinate on MS medium plates placed vertically in growth incubator. Pictures were taken from 10 day old seedlings and root length was measured using a standard ruler.

B) Root lengths for WT, *cbp60f*, CBP60f-2, CBP60f-3, CBP60f4, CBP60f5, and CBP60f-6. All the measurements were repeated three times and Standard error (SD) for mean was represented as small vertical bars on the top of histogram. Student's t-test was done, n=10 (** $P < 0.001$, * $P < 0.01$)

A.



B.

CHAPTER 4: DISCUSSION

Plants encounter various environmental stresses such as drought, salinity, low or high temperature, during their life cycle. These stresses limit the growth and productivity of crop plants to variable degrees depending on the onset time, duration, and intensity of stress. Environmental stress factors have been estimated to reduce crop yield up to 70% as compared to the yield under favorable conditions. It has been estimated that crops attain only about 25% of their potential yield because of the detrimental effects of environmental stress (Boyer, 1982). Traditional approaches for breeding crop plants with improved abiotic stress tolerance have so far met with limited success (Richards, 1996). Genetic engineering may offer an attractive alternative to traditional plant breeding. At present we have several examples of plants with improved or novel characteristics such as insect and herbicide resistance have been developed and are being cultivated (James, 2006). But so far no transgenic crop with improved abiotic stress tolerance has been developed that is a commercial success. Progress in breeding and genetic engineering for abiotic stress resistance is very slow because of the poor understanding of the molecular basis of abiotic stress signaling and effectors of stress tolerance. In order to create effective breeding and genetic engineering approaches to improve abiotic stress resistance of crops, it is imperative to understand the abiotic stress resistance mechanisms at molecular level.

In this context, brassinosteroids (BRs) are emerging as a new tool in plant science for the development of stress resistant crops. BRs are essential for normal growth and development of plants. BRs play multiple roles in the physiological and developmental processes of plants, including cell division and expansion, embryogenesis, vascular differentiation, photomorphogenesis, leaf development, flowering time, and senescence (Clouse and Sasse, 1998; Sasse, 2003; Clouse, 2008). The remarkable function of BRs lies in their ability to increase tolerance in plants against a wide range of environmental stresses (Krishna, 2003, Divi and Krishna, 2009a, b). The present study is part of the ongoing investigation of the molecular mechanisms underlying BR-mediated stress tolerance in the model plant *Arabidopsis thaliana* with specific focus on functional

characterization of two calcium signalling related calmodulin binding proteins belonging to the family CBP60 using reverse genetic approaches.

A microarray study aimed at identifying BR-regulated genes under no stress and stress conditions, resulted in identification of three Ca²⁺-signalling related genes belonging to the family of calmodulin binding protein 60(CBP60) (Krishna and coworkers, unpublished data). In this study, I further investigated the biological functions of the two CBP60 family members- CBP60f and CBP60g. The upregulation of *CBP60f* and *CBP60g* by BR and stress noted in publicly available AtGenexpress datasets and by qRT-PCR analysis together with the presence of potential BES1-binding sites in the promoter regions strongly suggest that these two genes are BRresponse genes in *A. thaliana*. CBP60f and CBP60g are the members of the CBP60 protein family in *A. thaliana*. *CBP60* gene family consists of eight members, *CBP60a* (At5g62570), *CBP60b* (At5g57580), *CBP60c* (At2g18750), *CBP60d* (At4g25800) *CBP60e* (At2g24300), *CBP60f* (At4g31000) and *CBP60g* (At5g26920) (Reddy et al., 2002). Recently, two closely related members *AtCBP60g* and *CaMBP50* (*AtCBP60h*, referred to as SARD1), were implicated in PAMP-triggered immunity and accumulation of SA, suggesting that these two family members play a positive role in plant immunity (Wang et al., 2009; Zhang et al., 2010; Wang et al., 2011). Both proteins were shown to bind DNA through their highly conserved central region, which exhibits no sequence similarity to other known DNA-binding proteins, and regulate expression of specific genes (Zhang et al., 2010). Thus, the CBP60 family likely represents a plant-specific family of transcription factors, with CaMbinding ability (Zhang et al., 2010). CBP60g was also shown as a positive regulator for drought tolerance (Dongli et al., 2012) and it has been shown that CBP60e and CBP60h overexpressing transgenic lines increased salt tolerance (Rahman, 2011). Collectively, these findings indicate that the CBP60 family is involved in abiotic and biotic stress responses in plants.

A study on loss of function for these genes achieved by using T-DNA insertion mutants for *CBP60f* and *CBP60g* demonstrates their importance in salt stress tolerance. *cbp60*, *cbp60g-1* and *cbp60g-2* showed severe effect on growth under high salt conditions. High sensitivity to salt was observed in all the three mutants when the seedlings were grown on

soil and watered with 150 mM NaCl (Figure 3.9). To check the effect of salt on germination, mutants and WT seedlings were allowed to germinate on MS plates at different concentrations of salt. In contrast to the salt sensitivity results on soil, *cbp60g-1* and *cbp60g-2* showed resistance to salt for seed germination compared to WT (Figure 3.8 A, B) and Surprisingly, the *cbp60g-1* and *cbp60g-2* mutants showed better germination and root phenotype on plates compared with WT under salt conditions (Appendix 4.2). The contradictory soil versus plate results with of *cbp60g* mutants were puzzling in the view of fact that *CBP60g* is strongly upregulated in response to salt and osmotic stress. It is possible that a compensatory mechanism is activated in *cbp60g-1* and *cbp60g-2* mutant seedlings when grown on synthetic media. It is possible that CBP60 family members may be upregulated in *cbp60g* mutant strains. Further investigation in the phenotypic differences is required to elucidate the role of CBP60g in salt stress. On the other hand, *cbp60f* mutant seed germination was drastically inhibited in high salt concentration compared to WT and quite clear that mutation in the *CBP60f* gene leads to salt sensitivity. To further continue the studies in elucidating the role of *CBP60f*, transgenic lines overexpressing *CBP60f* were generated and further analysed.

In *CBP60f* overexpressing lines, the expression of *CBP60f* transcripts are closely correlated with the root phenotype. All the *CBP60f* overexpressing lines showed increased root length phenotype compared to WT and mutant. The increase in *CBP60f* transcript expression increased the length of roots proportionally under normal conditions. CBP60f-4 and CBP60f-3 overexpressing lines with maximum expression of 9 fold and 7 fold increases over WT (Figure 3.10B) showed longer roots than other transgenic lines with 40.5 mm and 38.2 mm respective root lengths. Whereas the OE lines CBP60f-2, CBP60f-5 and CBP60f-6 with 5- fold, 3.3-fold, and 6- fold increase in expression showed almost similar root lengths of 28, 25 and 31 mm respectively (Figure 3.12). These changes in root length with the change in *CBP60f* expression clearly indicate the role of CBP60f in determining the root length in *A. Thaliana*.

In addition, roots of *CBP60f* overexpressing lines also showed increased salt tolerance compared to WT and *cbp60f*, where the length of root was taken into consideration to determine the salt tolerance (Figure 3.11). The root lengths for WT and *cbp60f* mutant

were highly affected in a negative manner showing a gradual decrease with increase in salt concentrations, whereas *CBP60f* OE lines showed improved root. The long root phenotype exhibited by *CBP60f* OE lines could be a reason for increased salt tolerance. In general, influx of Na^+ into the root system is counteracted by efflux of Na^+ to the soil solution, an active process likely to occur via a Na^+/H^+ antiporter system. In *Arabidopsis*, some salt stress responsive genes like *SOS3*, a calcium binding protein are believed to be involved in the activation of such Na^+/H^+ antiporters (Qiu et al., 2002) and yet uncharacterized Na^+/H^+ antiporters might also be involved in the efflux of Na^+ from the root system, as these systems appears to be expressed more in the stele of mature roots (Pardo et al., 2006). Since *CBP60f* is found to be a salt stress responsive gene, there could be a possibility for *CBP60f* to activate these Na^+/H^+ antiporter systems. The increased root length phenotype for *CBP60f* OE lines lends strength to this hypothesis. This could be one of the reasons behind the improved salt tolerance.

Interestingly, the *CBP60f* overexpressing lines showed better phenotype for growth under salt stress conditions compared to WT and *cbp60f* mutant (Figure 3.10A). Our preliminary data has shown two overexpressing lines, *CBP60f*-3 and *CBP60f*-4, with higher expression (7 fold and 9 fold over WT) exhibiting an increase in resistance to salt stress with 82% and 78% survival rates. Whereas, the other two lines, *CBP60f*-2 and *CBP60f*-5, with 6 fold and 3.5 fold increase in *CBP60f* expression compared to WT had shown almost equal resistance to salt with 68% and 64 % survival rates while WT and mutant has 48% and 4 % survival rates respectively after three weeks of 150 mM NaCl treatment (Figure 3.10C). Preliminary RT-PCR analysis revealed higher expression of the salt stress regulated genes *RD29* and *RD22* in *CBP60f*-4 overexpressing line under salt stress compared to WT (Appendix 4.1) These data together with increased survival rates and better root phenotype in overexpressing lines compared to WT and *cbp60f* under salt stress clearly supports a role of *CBP60f* in salt stress tolerance in the plants of *A. thaliana*. Still more studies are required to fully characterize the effects of *CBP60f* overexpression in different transgenic lines. It is possible that successful use of the transgene for a given phenotype is critically dependent on achieving an optimal level of expression for the particular phenotype.

The present study adds new dimension to the functions of these genes by demonstrating their role in salt stress tolerance and root growth. Bioinformatics analysis of the promoter region of *CBP60f* showed potential transcription factor binding sites that could mediate expression of *CBP60f* in response to BR exposure or stress. For instance, BR-mediated regulation of *CBP60f* may occur through potential BES1-binding and light-regulated expression may occur via factors binding to GATA elements. The ACE motif of *CBP60f* may function to regulate growth and root length. Subcellular localization results revealed that CBP60f is localized in both the cytoplasm and nucleus. Surprisingly the CBP60f protein does not contain typical nuclear localization signal (NLS). The protein-protein interactions between CBP60f and other proteins may facilitate its localization to the nucleus or a novel NLS may be present. This aspect could also be interesting to investigate further studies. The CBP60 family likely represents a plant-specific family of transcription factors, with CaMbinding ability (Zhang et al., 2010). This hypothesis is supported by the present results of nuclear localization of CBP60f which may bind to the specific DNA sequence on target genes.

Ca²⁺-signaling plays a fundamental role in plant growth and adaptation to various stress factors. The *A. thaliana* genome possesses a large repertoire of Ca²⁺-signaling proteins including calmodulins (CaMs), calmodulin-like proteins (CMLs), Ca²⁺-dependent protein kinases (CDPKs), calcineurin B-like proteins (CBLs) and CaMbinding proteins (CaMBPs) (Day et al., 2002; Bouche et al., 2005). The majority of known CaM-binding sites in CaMBPs consist of a stretch of 12– 30 contiguous amino acids with positively charged amphiphilic characteristics that tend to form an alpha-helix upon binding to CaM (Reddy and Reddy, 2004; Bouche et al., 2005). Ca²⁺/CaM is believed to bind to its targets mainly by hydrophobic interactions, with electrostatic interactions contributing to the stability of the CaM-target complex. The multiple sequence alignment for CBP60 proteins revealed that the CaM-binding motif in the CBP60 family is located at the C terminus of the proteins, but this motif is absent in CaMBP50 (SARD1) (Wang et al., 2011). It should however be noted that some CaM-binding proteins do not possess the typical CaMBD motifs, and thus, not all CaM-binding motifs have been identified as yet. Furthermore, interaction of different CaMs/CMLs with the same CaMBP may occur *in vivo* under different physiological conditions. For this reason, the observation that

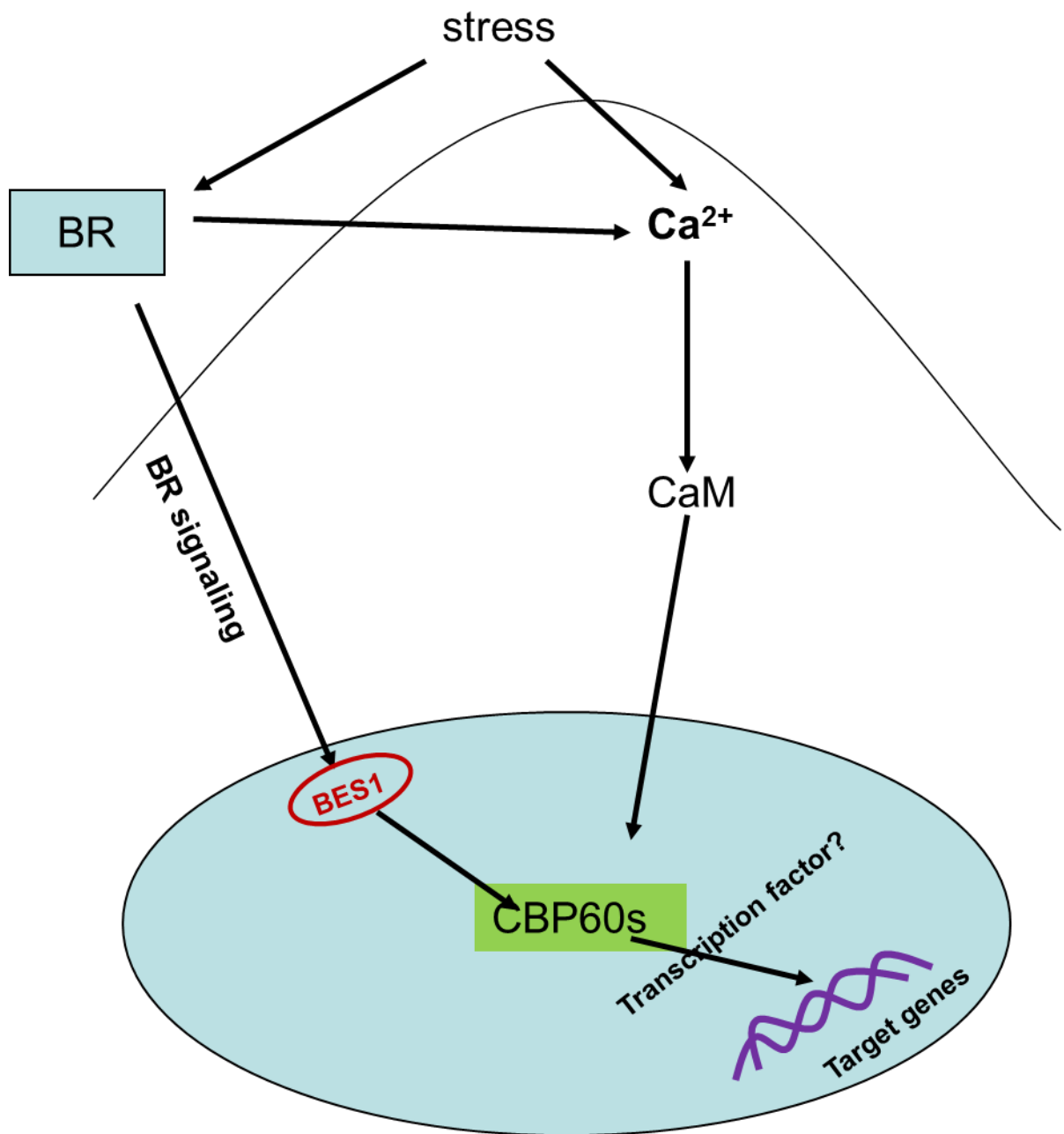
CBP60g can bind CaM (Wang et al., 2009), but that CaMBP50 lacks the ability to bind CaM (Wang et al., 2011), should be treated with caution.

Based on the present study, I have formulated a model for CBP60f/g signalling during stress. Under salt stress conditions, an increase in the Ca^{2+} levels activates the Ca^{2+} /CaM cascade and directly induces CBP60s. Alternatively, the induction of CBP60s may occur through BR signalling pathway where the stress induced BR signalling cascade activates transcription factor BES-1, which binds to the promoter sequences of *CBP60* genes and regulate their expression. Induced CBP60 may act as transcriptional factors by binding to the specific DNA sequences on stress related target genes. The proposed model is represented in a diagrammatic form (Figure: 4.1)

Salt stress tolerance and Ca^{2+} -signaling have previously been linked via calcineurin B-like proteins (CBLs), SOS3 and AtCBL1, which act as positive regulators of salt-stress tolerance (Zhu, 2000; Cheong et al., 2003). AtCaMBP25, a nuclear localized calmodulin binding protein was reported to negatively regulate the saltstress response in seed germination and seedling growth (Perruc et al., 2004). Increased sensitivity to NaCl-induced inhibition in germination was shown in transgenic lines overexpressing *AtCaMBP25*, whereas transgenic lines expressing an antisense transcript displayed greater resistance against inhibition of germination generated by D-mannitol and NaCl-induced osmotic stress (Perruc et al., 2004). The present study indicates that *CBP60f* acts as a positive regulator, whereas *AtCaMBP25* acts as a negative regulator of salt stress response. Therefore, reprogramming of responses occurs at different times during stress and possibly in different tissues, during environmental stress. In conclusion, a new BR response gene with functions related to growth, root length and stress responses have been identified in *A. thaliana* through the present study. Genetic manipulation of these genes in crop and bioenergy plants holds the possibility of simultaneously increasing yield and stress tolerance.

Figure 4.1: Proposed model for CBP60f function in stress signaling.

Under stress conditions, an increase in the calcium levels results in the activation of CBP60s directly by the Ca^{2+} -CaM cascade, or by the BR signaling pathway where BR activates the transcriptional factor BES1, which binds to promoter sequences of *CBP60* genes thereby regulates its expression. Induced CBP60s may act as transcriptional factors by binding to the specific DNA sequences on stress related target genes.



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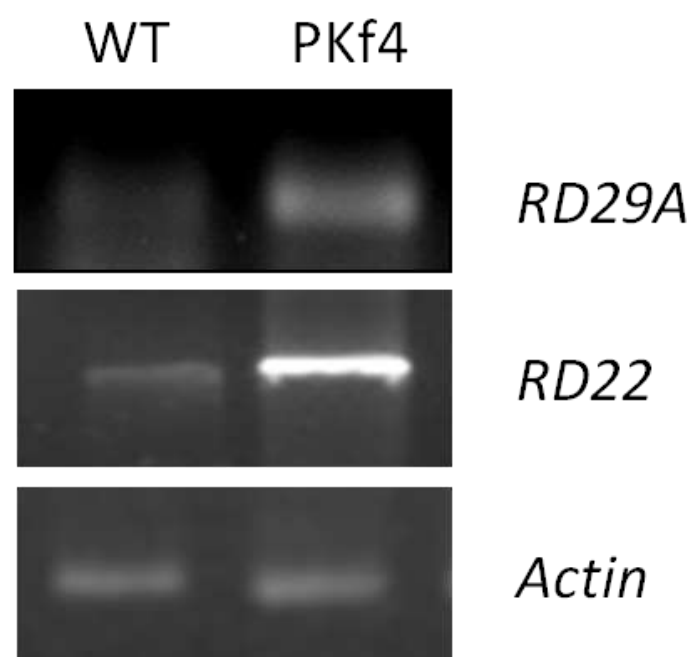
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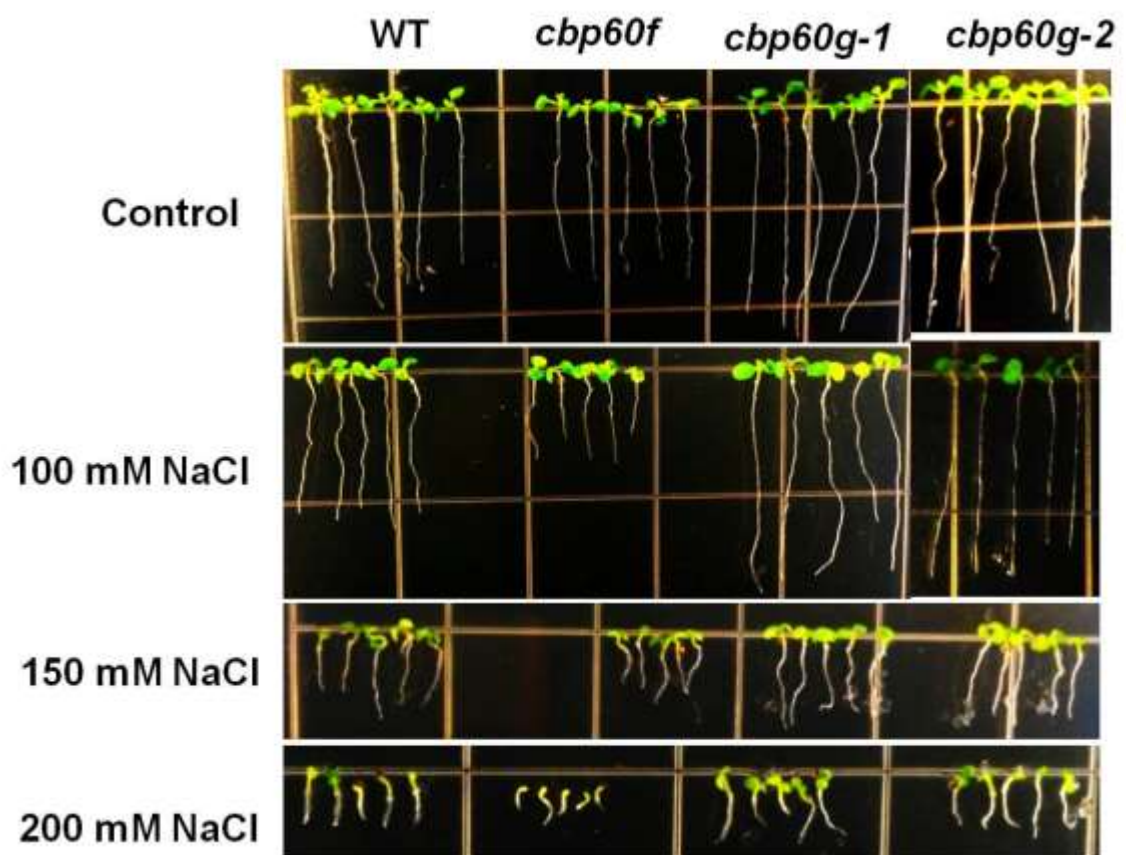
APPENDICES

Appendix 4.1: Expression of stress marker genes

RNA isolation and RT-PCR was performed to the leaves collected from WT and CBP60f-4 plants treated with 150 mM NaCl.



Appendix 4.2: Root phenotypes of WT, *cbp60f* and *cbp60g* mutant lines under different concentrations of salt



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